The occurrence of amoeba resistant bacteria (ARB) and Free-Living Amoeba (FLA) in samples from a wastewater treatment plant in Johannesburg, South Africa

Dissertation Submitted by

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DECLARATION OF INDEPENDENT WORK

I, Dr. Olivier Mwamba (Student number 200582682), do hereby declare that this research project, submitted to the University of Johannesburg, Faculty of Health Sciences for **Doctorate Technologyae in Biomedical Technology** , is my own independent work. This work has not been submitted before to any institution by myself, or to the best of my knowledge, any other person in fulfillment of requirements for the attainment of any qualification.

…….. …….

Dr. Olivier Mwamba Date

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ABSTRACT

The occurrence of amoeba resistant bacteria (ARB) and Free-Living Amoeba (FLA) in samples from a wastewater treatment plant in Johannesburg, South Africa

By Olivier Mwamba

Supervisor: Prof. D. Bartie

Co-supervisor: Prof. T.G. Barnard

Degree: Doctorate Technologiae In Biomedical Technology

Background: The continued presence of free-living amoeba (FLA) that harbour pathogenic amoeba resistant bacteria (ARB) is a growing concern internationally and is an issue not investigated in South Africa to date. This could lead to the pathogenic bacteria bypassing the purification process and be responsible for the outbreak of epidemics or lead to the infection among immune- compromised individuals. This study was carried out in one of the six wastewater treatment plants in Johannesburg, Gauteng to establish baseline data of ARB and FLA in wastewater treatment processes in the studied location.

Different types of amoeba harbour different types of ARB and in this study, we focused on *Acanthamoeba* as the FLA of choice. This study also included *Escherichia coli, Shigella, Salmonella, Vibrio cholera* as diarrhoea causing ARB and *Legionella*, *Mycobacterium avium*, and *Chlamydia species* as pneumonia causing ARB.

Objectives: The study objectives were to isolate the FLA from the treatment plant and to establish if *Acanthamoeba* species were present in these samples. Furthermore, it proposed to isolate the amoeba resistant bacteria found in these samples. Another objective was to assess the effectiveness of the current process to remove FLA and ARB from the water before it leaves the plant.

Methods: A seasonal sampling approach was used to collect the samples from each of the eight processes employed by this plant to treat the wastewater. The physico-chemical parameters (pH and temperature) of each sample were obtained at collection. Samples were concentrated using membrane filtration using cellulose-nitrate filtration method and FLA were cultured with the

amoebal enrichment methods. The ARB was cultured using selective media for each species of ARB under investigation. Microscopic investigation was conducted using the Giemsa, Gram and Ziehl-Neelsen stains. This study further investigated the effectiveness of direct PCR identification methods to identify the FLA (*Acanthamoeba*) and ARB's (*Mycobacterium avium, Vibrio. cholera, Legionella* and *Chlamydia*) found in these samples. Physico-chemical data were represented in tables showing means and ranges and it was further statistically analysed using the One Way Anova method. The culture and staining results were reported in frequency tables and no statistical analysis were performed on these.

Results: The temperature of the samples during the different seasons significantly changed, were as the pH were not different amongst the processes or the seasons. The study found that FLA were present in all processes and in all seasons in the samples from this plant. Only a small percentage of the samples tested positive for Acanthamoeba and this was mainly in autumn. Furthermore, the majority of bacteria found in all processes were extra cellular bacteria with a small percentage of the trophozoites and cysts presenting with intracellular bacteria. The Acanthamoeba cysts were presenting with intracellular bacteria most often, but a small percentage of round cysts also presented with intracellular bacteria. *Escherichia coli* and *Shigella* were the most abundant ARB found with Escherichia coli being present in all the samples tested as well as *Shigella* accept one specimen. Only one sample tested positive for *vibrio cholera* and this was in the exit process of the plant. Further, just over half of all the samples collected cultured positive for *Mycobacterium sp.*, but only one of these samples could be confirmed as M. avium via PCR analysis. Although PCR found several samples positive for *Chlamydia,* the majority of these was with round cysts and extracellular bacteria. The most concerning problem highlighted in this research is the fact that FLA's and ARB's, regardless of species, were found to be present in the effluent of the plant.

Conclusion: It is of utmost importance that the presences of FLA and ARB be included in the battery of tests used to monitor water safety and efficacy of treatment processes.

We found:

- FLA were present in this wastewater treatment plant and that they were not effectively removed by the treatment processes.

- That pathogenic ARB was present in the samples collected from this plant and in some cases are intracellular in the FLA found in the plant, thus surviving the treatment and persisting in the effluent of the plant.

Publications: To date, one publication has originated from the work. We are currently working on the second manuscript to be submitted for potential publication.

Muchesa P, Mwamba O, Barnard TG, Bartie C. (2014). Detection of free-living amoebae using amoebal enrichment in a wastewater treatment. *Biomedical Research International*, 2014, 10 pages. Article ID 575297, 10 pages, 2014. http://dx.doi.org/10.1155/2014/575297

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
AIDS	Acquired Immune Deficiency Syndrome
ARB	Amoeba Resistant Bacteria
BCYE	Buffered charcoal yeast extract
BOD_5	Biochemical Oxygen Demand
$CaCl_2$.	Calcium Chloride
$CaCl_2.2H_2O$	Calcium Chloride Dihydrate
$CaSO_4$	Calcium Sulphate
CNS	Central Nervous System
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DWAF	Department of Water Affairs and Forestry
Fig.	Figure
FLA	Free living amoebae
GAC	granula activated carbon
GAE	granulomatous amoebic encephalitis
h	Hour
H_2O	Water
HIV	Human Immunodeficiency Virus
kg	kiloGram

KH_2PO_4	Potassium Dihydrogen Phosphate
MAC	Mycobacterium avium complex
MCLs	Maximum Contaminant Levels
mg	MilliGram
Mg/ml	MilliGram per Milliliter
$MgSO_4\ 7H_2O\ \cdot$	Magnesium Phosphate Hepta hydrate
MIC	Minimal Inhibitory Concentration
MIL	Maximum Inhibition level
min	Minute
MLSS	Mixed-Liquor Suspended Solids
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium Chloride
NIOH	National Institute for Occupational Health
NNA-*E*	Non-nutrient agar-E.Coli plate
NNA	Non-nutrient agar
NTM	Non-Tuberculous Mycobacteria
PAM	Primary amebic meningoencephalitis
PAS	Page's amoebal saline
PCR	Polymerase Chain Reaction
RBC	Rotating biological contactors
SA	South Africa
SS	Suspended Solids
TB	Tuberculosis

TCBS	Thiosulfate citrate bile sucrose agar
TT	Treatment Technique
USA	United States of America
WHO	World Health Organization
WHRC	Water and Health Research Centre
XLD	Xylose lysine desoxycholate agar
ZN	Ziehl Neelsen
µg	MicroGram
µl	Microliter
µM	Micrometrer

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CHAPTER 1

1 INTRODUCTION AND LITERATURE REVIEW

1.1 BACKGROUND

The presence of free-living amoebae (FLA) in wastewater treatment plants raises serious concerns in the water industry due to the role these organisms play as potential pathogens and reservoirs of amoeba resistant microorganisms in water distribution systems.

The abundance and diversity of FLA in soil and water are influenced to a large extent by environmental conditions such as season, temperature, moisture, precipitation, pH and nutrient availability (Loret and Greub, 2010; Bonilla-Lemus *et al.*, 2013, Hsu, 2016, Tecon and Or, 2017; Inkinen *et al.,* 2019). FLA are abundant in biofilms and at soil/plant, plant/water and water/air interfaces (Coulon *et al.,* 2010; Garcia *et al.*, 2013, Bonilla-Lemus *et al.*, 2013; Scheid, 2014; Flemming and Wuertz, 2019). Free-living amoebae (FLA) are important predators of bacteria, fungi and algae (Berry *et al.,* 2010, Coskun *et al.,* 2013, Scheid, 2014). They feed by phagocytosis and digest their food in phagolysosomes. However, a number of bacteria have become resistant to, and are able to survive destruction in phagolysosomes, allowing them to propagate and eventually be released in the environment (Berry *et al.,* 2010, Ovrutsky *et al.,* 2013, Scheid, 2014, Hsu, 2016; Uribe-Querol and Rosales, 2017; Echeverria-Valencia, Flores-Villalva, and Espitia, 2017). These amoeba resistant bacteria (ARB) can survive inside resistant amoebal cysts making them difficult to detect with traditional methods as these methods only test for extracellular bacteria such as faecal coliform and *Escherichia coli*. There are also health implications to these ARB since they can survive the chlorine levels commonly used to treat water whilst inside their amoebal hosts (Berry *et al.,* 2010; Loret and Greub, 2010; Ovrutsky *et al.,* 2013, Scheid, 2014; Inkinen *et al.,* 2019).

Amoeba resistant bacteria are an important cause of disease. They are transmitted through water distribution systems and may cause infection either through inhalation of aerosols, through direct contact or through ingestion. (Gebert *et al.*, 2018) This puts populations with high numbers of immuno-compromised individuals at high risk (Fukumoto *et al.*, 2010; Coskun *et al.,* 2013; Bentham and Whiley, 2018).Taking the high incidence of tuberculosis (TB) and human immunodeficiency virus (HIV) infections in South Africa into consideration, the number of immuno-compromised persons are increasing and with it the risk of infection with ARB (Cronje *et al.*, 2013; SANAC, 2017).

A study by Keddy *et al.* (2012) demonstrated that HIV patients had a 4.1 times higher risk of dying from systemic shigellosis and that 67% of all cases of systemic shigellosis were found

in HIV positive patients (Okeke, 2009). Without appropriate and timely interventions this may have detrimental effects, not only in the water treatment industry but also on the economy of the country as it impacts on the ability of these individuals to be productive and support themselves and their families.

Although some studies were done in South Africa on *Legionella* (Bartie and Klugman, 1997; Singh and Coogan, 2005; Wolter *et al.*, 2016), *Chlamydophila pneumoniae* (Bartie and Klugman, 1997 and Black, 2008; Crowther-Gibson *et al.,* 2011) and environmental *Mycobacterium* species (Corbett *et al.,* 1999a; Corbett *et al.,* 1999b and Black, 2008; Crowther-Gibson *et al.,* 2011), their survival within and relationship with free living amoebae has not been studied thoroughly in South Africa.

As for the other bacteria investigated in this study all of them has been extensively studied in the South African context during outbreaks of disease and in epidemiologic studies, but none of them have been studied in relation to their potential ARB status and relationship to FLA in the South African water quality context.

A study previously conducted in South Africa showed that 36% of gold miners were positive for *Legionella pneumophila serogroup* (SG) 1-4 antibodies compared to 16% of hospitalised pneumonia patients and 10% of factory workers (Bartie and Klugman, 1997). However, the seroconversion (fourfold increase in antibody titre over a certain period) in 18% of the mine workers occurred over a period of 6 months while 14% of the pneumonia patients showed seroconversion within 2-4 weeks. In further studies conducted between 2012 and 2014 amongst patients that presented with severe respiratory illness, Legionellae were found in 1.2% of cases investigated as a co-infection of tuberculosis (Wolter *et al.* 2016). This trend was similar for *Chlamydophila pneumoniae* where 66% of mine workers developed antibodies as opposed to 50% and 22% for pneumonia patients and factory workers respectively. Again, seroconversion was demonstrated in 17% of miners over a period of 6 months and in 22% of pneumonia patients in 2 - 4 weeks (Bartie and Klugman, 1997 and Black, 2008). Other studies conducted in South Africa showed that Legionellae were present in 82% of industrial water samples tested with 54% of these having a colony count of above 10^3cfu/ml (Bartie *et al.,* 2001; Bartie *et al.,* 2003; Hamilton *et al.,*2019). Later studies done on rainwater tanks showed that 73% of samples tested positive for *Legionella,* (Dobrowsky *et al.*, 2014; Dobrowsky, Khan and Wesaal, 2017; Hamilton *et al.,*2019). In a study done in the late 80's (Tobiansky *et.al.,* 1986) a seasonal pattern of Legionaires' disease was found, therefore this study extended it's sampling to include all seasons. This is also the case with cholera

(Bateman, 2009) and the other diarrhoeagenic bacteria; most of these seasonal variations were related to rainfall (Emch *et al.*, 2008; Estrada-Garcia *et al.*,2009; Hamilton *et al.,* 2019). During the rainy season and especially after the first rain decaying matter and faecal contaminants are washed into the rivers and water supply via surface water runoff and stormwater collection systems.

Studies done by September *et al.* (2004) and Ovrutsky *et al.* (2013) suggested that the currently accepted microbial water quality parameters fail to detect the potential health risk posed by non-tuberculous mycobacteria (NTM) in distribution networks. (Gebert *et al.* 2018) This conclusion was reached when results from the September *et al.* (2004) study showed that 18% of the 78 biofilm samples they tested from urban and semi-urban sources were positive for NTM, with similar percentages reported by Ovrutsky *et al.* (2013) and Gebert *et al.* (2018)

Hence, this project assessed the occurrence of free-living amoebae (FLA) potentially containing amoeba resistant bacteria (ARB) in samples from a wastewater treatment plant in Johannesburg, South Africa during all seasons and in the different stages of the wastewater treatment process. The samples were analysed using amoebal enrichment techniques. The influences of season, temperature, and pH as co-variants were also assessed.

1.2 THE STUDY

1.2.1 Study Motivation

The occurrence of free-living amoebae (FLA) potentially harbouring amoeba resistant bacteria (ARB) in wastewater treatment plants in greater Johannesburg, South Africa requires particular attention. This is due to the threat posed by these potentially pathogenic microorganisms to workers in wastewater treatment plants as well as to communities living in the surroundings of wastewater effluents, rivers, and dams. Moreover, rapid detection of waterborne pathogens which occur at low levels in environmental waters are essential for the protection of public health.

South Africa is a water stressed country; the quality of wastewater is therefore particularly important. For example, Gauteng accounts for 46% of the population involved in industry related vocations, such as mining, manufacturing, construction, and transport in South Africa (StatsSA, 2014). In 2013, most of Gauteng residents (95.9%) had access to piped water and proper sanitation. Taking this into account and the fact that the population growth of the

province due to migration and new births is the highest in the country with the number of households increasing by 52% from 2003 to 2013 (StatsSA, 2014) the burden to provide proper reclamation of wastewater is a growing challenge. Although wastewater is tested regularly for faecal indicator organisms (Huws *et al.* 2006; Steinberg and Levin. 2007; Lamoth and Greub, 2010 and Loret and Greub, 2010), this may not be sufficient as shown by Dobrowsky *et al.*, 2014 and by preliminary studies that indicated the presence of high numbers of FLA and ARB in water samples collected from the wastewater treatment plant under investigation, even after chlorination.

This study intended to ascertain whether there was a need to isolate and identify pathogenic ARB from wastewater which can cause disease in the general public and in workers from the treatment plant (Black, 2008; Ashbolt, 2015) and if there are ARB present. Furthermore, the study intended to identify at which stages of the wastewater treatment process the amoebae and bacterial loads are the highest and whether the prevalence is influenced by season.

This study therefore aimed to highlight the importance of amoebal co-culture and enrichment techniques to test wastewater for the presence of several emerging and re-emerging amoeba resistant waterborne pathogens in wastewater samples collected from a treatment plant in Johannesburg South Africa.

1.2.2 The research question

Is there an association between the presence of free-living amoeba in a wastewater treatment plant in Johannesburg South Africa, and the occurrence of amoeba resistant bacteria that could be of importance to human health?

1.2.2.1 Hypothesis to solve the research question

Free living amoebae occur throughout the wastewater treatment process. These amoebae contain amoeba resistant bacteria (ARB) that are pathogenic to workers in the wastewater treatment plant and to susceptible people of the surrounding communities.

1.2.3 Aims

The aims of the study were to establish whether certain FLA and ARB are present in various stages of wastewater treatment, to study seasonal differences in their occurrence and to provide recommendations to management on decreasing the levels of these organisms in the treatment process if they are present.

1.2.4 Objectives

The study objectives were to:

- Isolate FLA from wastewater samples
- Establish whether potentially pathogenic *Acanthamoeba* species are present in wastewater samples
- Isolate amoeba resistant bacteria
- Identify amoeba resistant bacteria to genus and species level.
- Determine whether the presence of FLA and ARB are seasonally dependent.
- Assess the effectiveness of the current treatment process to remove FLA and ARB.
- Make recommendations to the wastewater treatment plant regarding optimising the treatment process.
- Report/publish results.

1.3 LITERATURE REVIEW

1.3.1 Definitions

1.3.1.1 Wastewater:

Wastewater is all water in which the quality has been decreased as a result of contamination due to human activity in and around such water. It includes industrial, agricultural, and municipal effluent. (Summarised from Enviromental-Expert http://www.environmental-expert.com/services/wastewater-definition-of-117681)

1.3.1.2 Sewage

Sewage is a subset of wastewater which is contaminated with urine and faeces and is carried via sewers to the treatment plant. (Adapted from the Cambridge dictionary http://dictionary.cambridge.org/dictionary/british/sewage)

1.3.2 South African wastewater treatment legislation, standards, and regulations

The management and treatment of wastewater and the discarding and use of effluent are regulated under different Acts. These Acts reside under different governmental departments. The main Acts governing this process are the National Water Act, 1998 (Act no 36 of 1998) and the National Water Services Act, 1997 (Act no 108 of 1997) which are implemented by the Department of Water Affairs (DWA) with the implementation of all resolutions devolved to provincial and local governments. The legislation does not contain comprehensive standards, but these are set out in guidelines by the DWA or at the South African Bureau of Standards (SABS) for the different types of waters to be used.

The latest draft guidelines available on their web site (www.dwa.gov.za) states that the safety of the effluent must be verified with the following parameters: faecal coliforms (per 100 ml), pH, electrical conductivity (mS/m), Chemical oxygen demand (mg/l), ammonia as nitrogen (mg/l), suspended solids (mg/l), nitrate/nitrite as nitrogen (mg/l), free chlorine (mg/l) and ortho-phosphate as phosphorous (mg/l). No reference is made to FLA or ARB in any of the water regulations or guideline (SANS 241-1, South African Water Quality Guidelines, 2015 SANS 241-1, 2015. Drinking Water Specification, Edition 2. South African National Standard Drinking water. Pretoria.) looked at, the closest reference to free living amoeba is reference made of protozoans in the domestic water use guidelines.

They further go on to state that the microbiological verification must be designed and conducted in such a way as to best detect any possible contamination. However, they again do not address the risk of free-living amoebae in any previously published regulations, guidelines or standards in South Africa.

1.3.3 Wastewater uses.

One of the most effective ways to dispose of wastewater is to use it for irrigation although it can be used for other agricultural, domestic, industrial, and recreational purposes as well. However, municipal wastewater needs some level of treatment before it can be used for domestic purposes such as irrigation.

Although there are numerous uses for the different types of wastewater, it suffices to state that in the scope of this thesis the wastewater investigated, and the effluent generated from it was released into environmental water bodies. The use of this water by communities, industries and agricultural bodies were outside the scope of this study, because only about 14% of wastewater in South Africa is re-used without prior treatment by the agricultural and

municipal sectors that use it for the irrigation of crops and public open spaces such as golf courses and sports fields. The indirect re-use of water however is well established in South Africa where the treated wastewater is released into the environment and downstream taken out and treated for domestic use. (Van Niekerk and Schneider, 2013)

1.3.4 Wastewater Treatment

Wastewater treatment is the process that allows effluents resulting from human and industrial activities to be treated and disposed of in a way which would not harm human health or endanger the surrounding environment and wildlife. The aim of wastewater treatment is to remove as much as possible organic and suspended material content to reduce environmental pollution. The removal of pathogens during wastewater treatment is complex and may be very costly, however it is one of the most important concerns in providing quality water to consumers and population. Proper design and operation of wastewater treatment plants are therefore essential (Hillman, 1988; Abdel-Raouf *et al.,* 2012). Despite all the necessary precautions in producing quality water free of health hazards, this can still pose challenges as microorganisms may show resistance towards the treatment processes, thus the imminent threat to humans.

The ideal wastewater treatment protocol should comply with the microbial and chemical standards and regulations of the area where it is situated, should be cost effective and should require minimal operational oversight and maintenance (Arar, 1988; Utsev and Agunwamba, 2012). Especially in developing countries where the expertise to operate complex systems is often not available and high costs should be avoided wherever possible, some locations will be better served with the re-use system accepting a lower grade of effluent rather than to apply advanced treatment processes to attempt to meet first world standards (Arar, 1988; Utsev and Agunwamba, 2012). Although none of these authors define what is meant by low-grade of effluent it is mentioned that the removal of pathogenic organisms is always important especially where such re-use involves proximity to human populations such as irrigation schemes.

1.3.5 Wastewater treatment processes.

An figure showing a basic configuration of a treatment plant is shown in Figure 1.1 below. Wastewater treatment entails a combination of processes and operations of physical, chemical, and biological nature, to clear the effluent of solids, organic matter, and other potentially harmful substances and in some cases nutrients that can stimulate algae and bacterial growth. The terms preliminary, primary, secondary, and tertiary and/or advanced treatment are used to describe different levels of wastewater treatment and indicate increasing levels of treatment (Al-Rekabi *et al.,* 2007; Abdel-Raouf *et al.,* 2012; Qurie *et al.*, 2015). A conventional wastewater treatment diagram is shown in Figure 1.2.

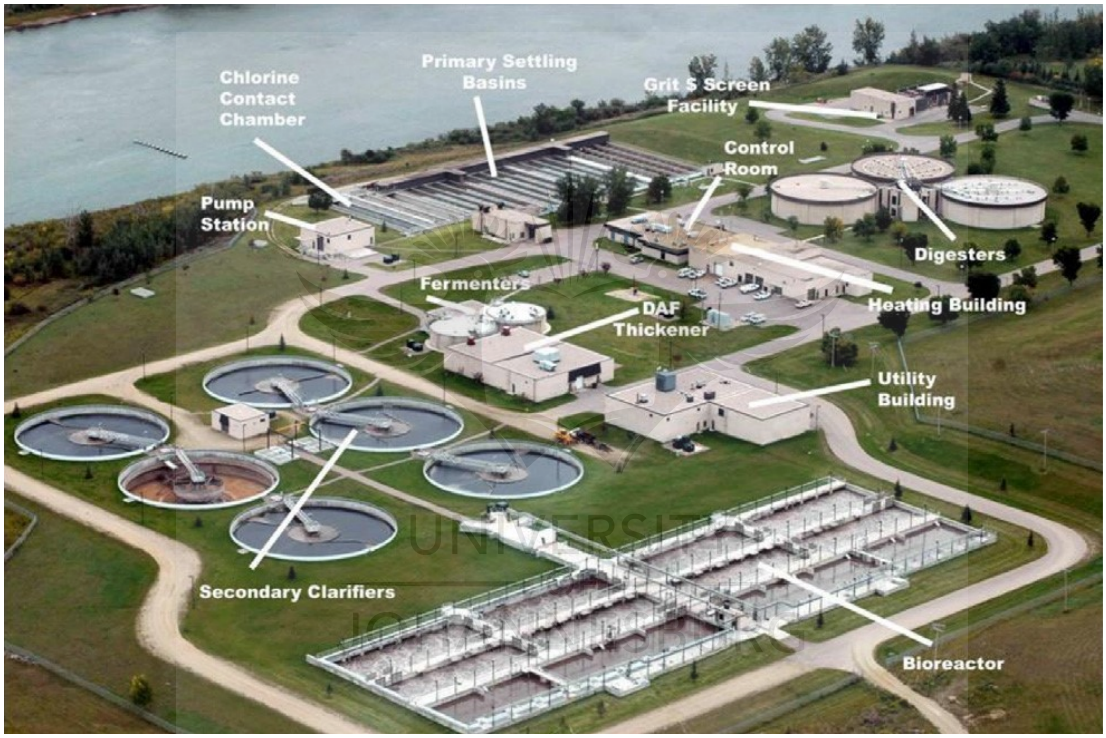


Figure 1.1: Wastewater treatment station model. (DAF-Dissolved Air Flotation) (Abdel-Raouf et. al., 2012)

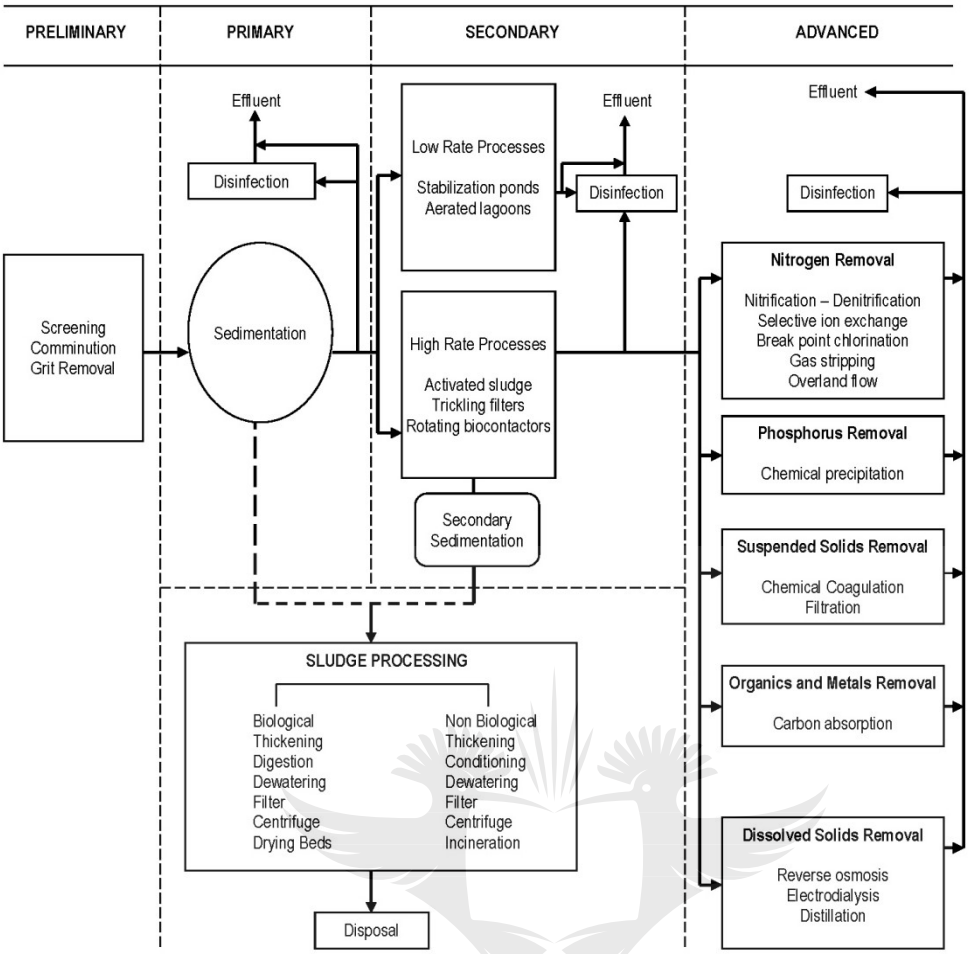


Figure 1.2: Generalized flow diagram for municipal wastewater treatment (Asano *et al.* 1985; http://www.fao.org/3/t0551e/t0551e0j.gif (last accessed 2020)

1.3.5.1 Preliminary treatment

Preliminary wastewater treatment consists of removing coarse solids and other large materials that is often present in untreated wastewater. This initial process typically includes coarse screening and grit removal. In large treatment plants, organic solids are prevented from settling by using water or air flowing through the grit chamber at high velocity (Metcalf and Eddy, 2003; Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012; Qurie *et al.*, 2015). In small wastewater treatment plants, comminutors (machines that cut solids present in raw sewage into small pieces in preparation for primary treatment) are often combined with coarse screening to reduce the size of large particles to be removed as sludge in subsequent treatment processes. Flow measurement devices such as standing-wave flumes, are regularly included as part of the preliminary treatment stage (Metcalf and Eddy, 2003; Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012; Qurie *et al.*, 2015).

1.3.5.2 Primary treatment

The primary treatment process removes settleable organic and inorganic material by sedimentation and floating material (scum) by skimming. It is estimated that primary treatment removes about 25 to 50% of the biochemical oxygen, 50 to 70% of the total suspended solids (SS), and 65% of the oil and grease present in the waste (Al-Rekabi *et al.*, 2007). Some organic components such as nitrogen, phosphorous and heavy metals linked with solids are also removed during the primary sedimentation process (Metcalf and Eddy, 2003; Abdel-Raouf *et al.,* 2012, Qurie *et al.*, 2015) (see Figure 1.2).

In terms of design, primary sedimentation tanks are round or rectangular basins, normally three to five meters deep, with a hydraulic retention period of between two to three hours. Sludge rakes remove settled solids (primary sludge) from the bottom of tanks to a central well from where it is pumped to sludge processing units. Scum is removed by sweeping it across the tank surface using water jets or by mechanical means from where it is also pumped to sludge processing units (Metcalf and Eddy, 2003; Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012; Qurie *et al.*, 2015).

1.3.5.3 Secondary treatment

The remaining organics and suspended solids are removed during secondary treatment by an aerobic biological treatment process. Aerobic biological treatment (Figure 1.3) refers to the removal of biodegradable dissolved and colloidal organic material by aerobic microorganisms (principally bacteria) in the presence of oxygen. These organisms metabolize the organic matter in the wastewater producing more microorganisms and inorganic end-products such as CO_2, NH_3, and H_2O (Metcalf and Eddy, 2003).

Although there are a number of different aerobic treatment processes, they only differ in the way that oxygen is supplied to the microorganisms and in the rate at which the organisms metabolize the organic matter (Metcalf and Eddy, 2003). In high-rate (small, fast) processes there are relatively small reactor volumes and high concentrations of microorganisms compared with low rate (large, slow) processes. Therefore, in high-rate systems the growth rate of new organisms is much higher due to the well-controlled growth conditions.

In order to produce clarified secondary effluent, the microorganisms have to be removed from the treated wastewater by sedimentation. This is done in sedimentation tanks similar to those described previously under primary sedimentation and are designated secondary clarifiers.

The sludge produced during secondary sedimentation is called secondary or biological sludge and is usually added to the primary sludge for sludge processing.

Common high-rate processes that could be employed during secondary treatment include trickling filters or biofilters, oxidation ditches, and rotating biological contactors (RBC) and the activated sludge processes (see Figure 1.2). Where municipal wastewater contains high levels of organic material from industrial sources, two of these processes are usually combined in series (e.g., biofilter followed by activated sludge) as part of the secondary treatment (Metcalf and Eddy, 2003; Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012; Qurie *et al.*, 2015).

1.3.5.3.1 Activated Sludge

The activated sludge process takes place in the dispersed-growth reactor which is an aeration tank or basin containing the mixed liquor which is a suspension of the wastewater and microorganisms. Aeration devices vigorously mixes the contents of the aeration tank at the same time supplying oxygen to the biological suspension (Metcalf and Eddy, 2003; Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012; Qurie *et al.*, 2015). Submerged diffusers that are designed to release compress air and mechanical surface aerators that introduce air by agitating the liquid surface are the more commonly used aeration devices. Wastewater is retained in the aeration tanks for between 3 to 8 hours but can be longer with high BOD_5 containing wastewaters. The secondary effluent is produced after the aeration step, when the microorganisms are removed from the liquid by a sedimentation process and the liquid is clarified. A high mixed liquor suspended solids (MLSS) level is maintained by recycling a portion of the biological sludge to the aeration basin. The rest is removed from the process and further processed by the sludge processing steps to maintain a relatively constant concentration of microorganisms in the process. Several other variations of the basic activated sludge process, such as extended aeration and oxidation ditches has been reported (Metcalf and Eddy, 2003; Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012; Qurie *et al.*, 2015).

1.3.5.3.2 Trickling Filters

Trickling filters also called biofilters is a basin or tower containing support media which can be materials such as stones, plastic shapes, or wooden slats. Wastewater continuously flows over the media allowing microorganisms present in the wastewater to attach and form a biological layer or fixed film on top of the media (Metcalf and Eddy, 2003 Al-Rekabi *et al.*,

2007; Abdel-Raouf *et al.,* 2012). These layers then allow the organic matter from the wastewater to diffuse into the film and be metabolized by the microorganisms. The relative temperatures of the wastewater and ambient air facilitate the natural flow of air either up or down through the media which supply the oxygen to these films. The biofilm increases in thickness as new organisms grow, which periodically causes portions of the biofilm to separate from the media. This sloughed biofilm material is removed from the liquid in the secondary clarifiers and removed to sludge processing units. The portion of the secondary effluent which is the liquid from the secondary clarifier is often recycled to the biofilter to improve hydraulic distribution of the wastewater over the filter (Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012).

1.3.5.3.3 Rotating Biological Contactors

Rotating biological contactors are fixed-film reactors similar to biofilters in so far as that organisms are attached to support media. In the rotating biological contractors, the support media are slowly turning discs which are partially submerged in shallow flowing wastewater within these reactors. The biofilm obtain oxygen from the air when the film is out of the water and from the liquid when submerged in the water, as oxygen is introduced into the wastewater by surface turbulence generated by the discs' rotation. Sloughed biofilm is removed in similar manner as was described for biofilters (Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012).

High-rate biological treatment processes, in conjunction with the primary sedimentation processes typically remove about 85 % of the BOD_5, SS and some of the heavy metals initially present in the untreated wastewater. Activated sludge on the other hand normally produces effluent of slightly higher quality, as far as these constituents is concerned, than biofilters or RBCs. (Metcalf and Eddy, 2003; Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012). When these processes are coupled with a disinfection process, this protocol can provide substantial but not complete disinfection of bacteria and virus. However, a very small amount of phosphorus, nitrogen, non-biodegradable organics, or dissolved mineral is removed during these processes (Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012).

1.3.5.4 Sludge processing

Primary and secondary sludge are combined and further processed by biological and non-biological processes as shown in Figure 1.2. The primary focus of sludge processing is four-fold: (1) stabilisation, (2) weight reduction, (3) reduction of pathogens and (4) improving the

characteristics of the sludge for further use (Abdel-Raouf *et al.,* 2012). One of the major processes that extends and comply in part to all focus areas is the digestion process.

1.3.5.4.1 Digestion

The digestion process uses anaerobic and facultative aerobic bacteria to metabolize the organic material in the sludge, thereby reducing the volume of sludge for ultimate disposal as illustrated in Figures 1.3 and 1.4. This assists in stabilising the sludge (non-putrescible) and improving its dewatering capabilities. Aerobic digestion is performed when the sludge in continuously aerated over a long period of time, which causes the microorganisms to reduce the biological degradable organic matter. This process requires energy, and the duration necessitates the provision of extra reactor volume which makes this process expensive to run see Figure 1.4.

Therefore, the most often used form of digestion is anaerobic digestion and is performed in anaerobic digesters which are covered tanks, typically seven to fourteen meters deep and can be heated or not heated. There are roughly 22 different types of anaerobic digesters (Grant *et al.*, 2002) in use in the world and it is not within the scope of this work to go into detail as to their design or function. A diagram of the process can be found in Figure 1.3. Therefore, the time that sludge will spend in a specific digester depends on the type and design of the digester and may be as little as 10 days for high-rate digesters (well-mixed and heated) to as long two months or longer in standard-rate digesters. During this process recoverable gas containing about 60 to 65% methane is produced that could be used as an energy source if the process is heated (Metcalf and Eddy, 2003; Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012). Small sewage treatment plants process sludge in a variety of different ways which include aerobic digestion, storage in sludge lagoons, direct application to sludge drying beds, in-process storage (as in stabilization ponds), and land applications.

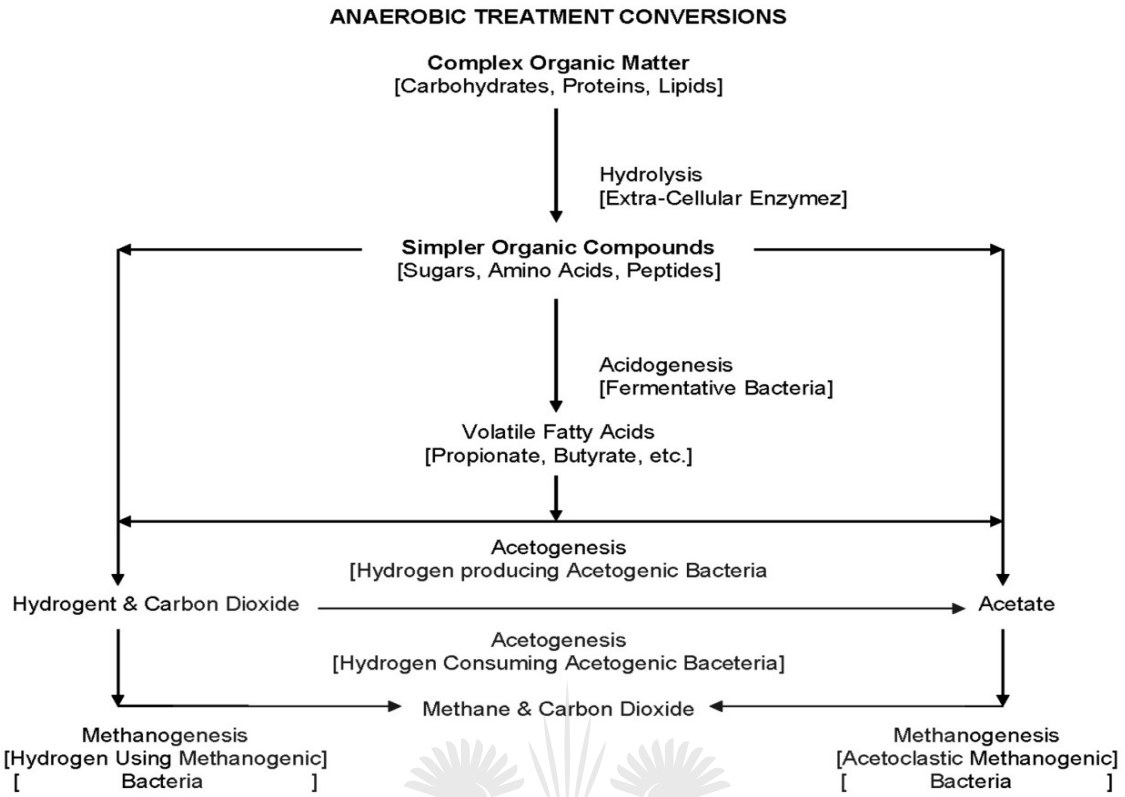


Figure 1.3 Anaerobic treatment procedure http://www.fao.org/3/t0551e/t0551e0k.gif (Accessed 6 October 2020)

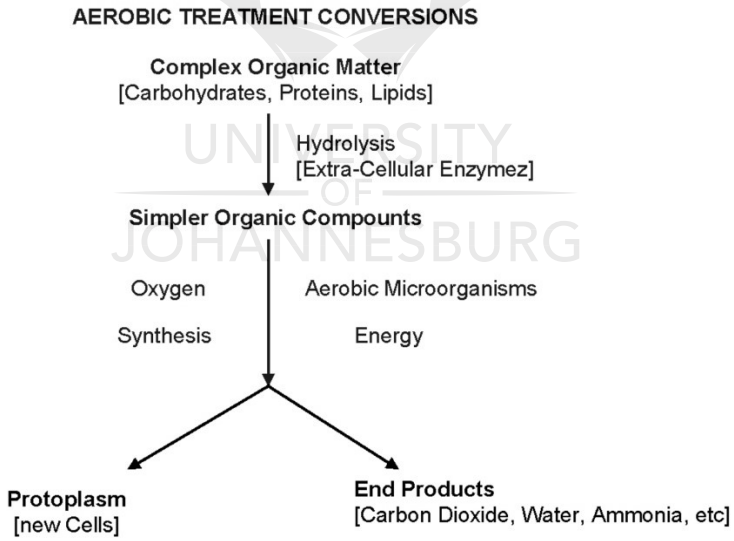


Figure 1.4 Aerobic treatment procedure http://www.fao.org/3/t0551e/t0551e0k.gif (Accessed 2020)

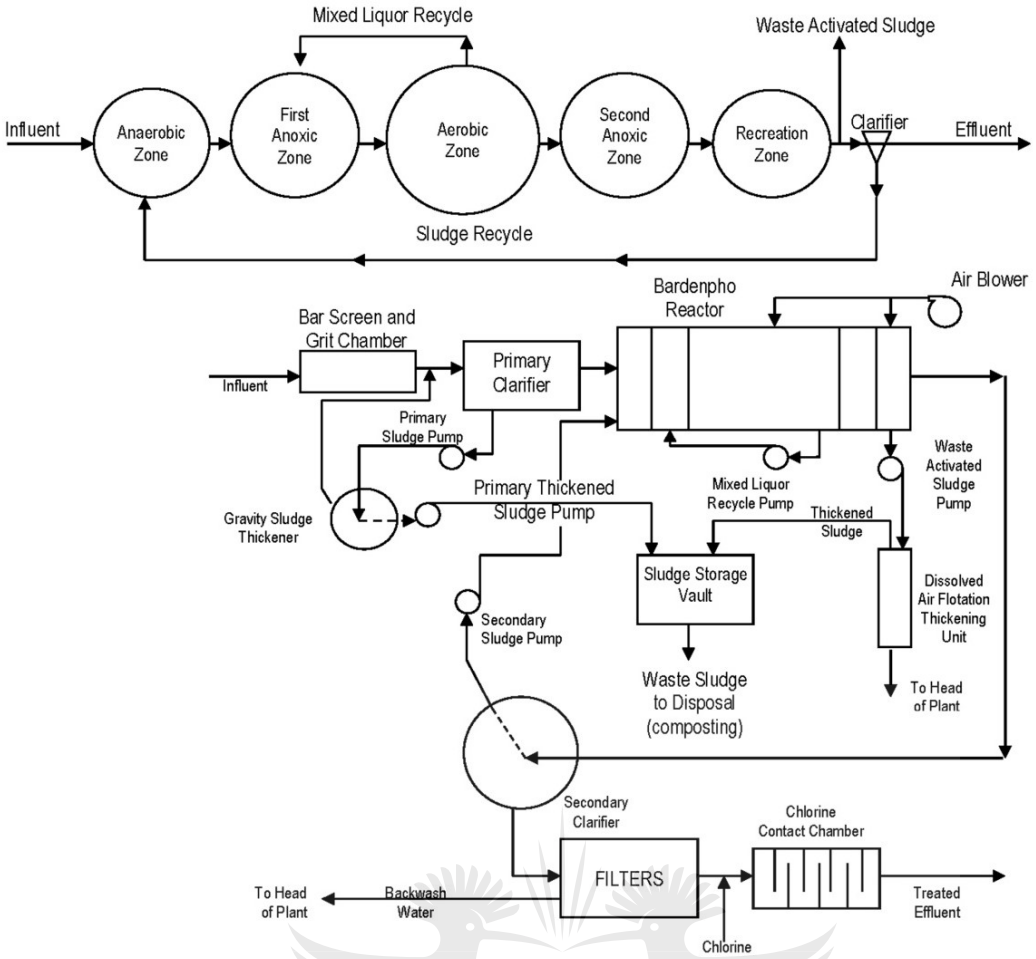


Figure 1.5: Simplified flow diaGram of Bardenpho-plant (Asano *et al.* 1985; http://www.fao.org/3/t0551e/t0551e0l.gif) (Last accessed 2020)

1.3.5.5 Advanced treatment

Advanced wastewater processes (also referred to as tertiary treatment) are called upon whenever specific wastewater components which have not been removed during secondary treatment have to be removed. Individual treatment processes are necessary for the removal of phosphorus, nitrogen, suspended and dissolved solids, refractory organics, and heavy metals. (Abdel-Raouf *et al.,* 2012). In some instances, the three treatment processes are partially or completely combined depending on the characteristics of the waste and the treatment plant, for example when chemicals are added to primary clarifiers or aeration basins to remove phosphorus or in the case of overland flow treatment of primary effluent (Metcalf and Eddy, 2003 Al-Rekabi *et al.,* 2007; Abdel-Raouf *et al.,* 2012).

The removal of nitrogen and phosphorus requires an adaptation of the activated sludge process and an example of this approach is found at the 23 Ml/d treatment plant commissioned in 1982 in British Columbia, Canada (World Water 1987). The first process used is to stress the bacteria responsible for removing the phosphorus by low oxygen

reduction to release more phosphorus to the system, which disturbs the equilibrium. Then when the effluent passes to the aerobic zone where oxygen and phosphorus is in abundance the bacteria now absorb much more phosphorus than they need for their normal biological functions. This allows the phosphorus to be removed from the system with the activated sludge waste (Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012).

When the wastewater enters the plant, the nitrogen component is almost completely in the form of ammonia, this is changed in the third aerobic zone where nitrates and nitrites are formed. For complete removal this effluent is returned to the first anoxic zone where bacteria process the nitrates to nitrogen gas and the nitrates that is not recycled in this process is then used for bacterial respiration in the second anoxic stage. (Al-Rekabi *et al.,* 2007; Abdel-Raouf *et al.,* 2012).

Where the risk of public exposure to the recycled water or residual components is high, the eventual aim of the wastewater treatment plant is to reduce the probability of the population being exposed to enteric viruses and other pathogens. Suspended and colloidal solids in the water inhibit proper disinfection of viruses. Therefore, solids must be removed by advanced treatment processes prior to the attempting the disinfection step. The proper sequence of treatment is believed to be the following: secondary treatment process followed by chemical coagulation, sedimentation, filtration, and disinfection. This level of treatment protocol is tailored to create an effluent free of most detectable pathogens (Al-Rekabi *et al.,* 2007; Abdel-Raouf *et al.,* 2012).

1.3.5.6 Wastewater Disinfection

Disinfection refers to the reduction and partial destruction of pathogens to acceptable limits to reduce their impact on human health. In most cases, faecal contamination is the major cause of microbiological health hazards associated with water consumption (Amahmid *et al.*, 2002; George *et al.*, 2002; Hamilton *et al.,*2019). Faecal contaminated water may contain *Escherichia coli, Shigella* species*, Salmonella* species and *Vibrio cholerae* which are the causal agents for respectively diarrhoea, dysentery, typhoid fever, and cholera. Domestic wastewater can further contain viruses, causing illnesses such as meningitis and hepatitis, as well as parasitic protozoans and helminths (Abdel-Raouf *et al.,* 2012; Agensi *et.al.*,2019).

Findings from Dean and Lund (1981) indicate that although 40 - 70% of coliforms can be eliminated with primary treatment, the use of biological processes like trickle filters and activated sludge may remove up to 99% of the pathogenic microorganisms (Al-Rekabi *et al.*,

2007; Abdel-Raouf *et al.,* 2012). However, further disinfection is usually required to ensure the safety of treated wastewater for reuse and human consumption (Al-Rekabi *et al.*, 2007; Abdel-Raouf *et al.,* 2012; Agensi *et.al.,* 2019). Even after disinfection some pathogens may still be present in treated wastewater in low numbers.

1.3.6 pH and Temperature in wastewater

The pH is a measure of the concentration of hydrogen ions in a solution. The pH is extremely important in biological wastewater treatment because the microorganisms remain sufficiently active only between pH 6.5 and 8.0. However, amoebae have a broader pH range (5.6 to 8.4) which makes them ideal carrier of the ARB (Sakran *et.al.*, 2019). Outside of this range, pH can inhibit or completely stop biological activity. Nitrification reactions are especially pH sensitive. Biological activity declines to near zero at a pH below 6.0 in acclimated systems (Srinu and Pydi, 2011; Wu *et al.,* 2013; Okaiyeto *et.al.* 2016). The same is true about the temperature of the water as most bacteria will only optimally grow between 10 to 20°C and temperatures outside these ranges will stress the organisms and they might either encyst or become dormant (Okaiyeto *et.al.* 2016).

1.3.7 Free living amoebae (FLA) in wastewater treatment

The importance of FLA in the wastewater treatment industry cannot be overlooked. Wastewater treatment is generally to allow human and industrial effluents to be disposed of without danger to human health or unacceptable damage to the natural environment. However, the presence of FLA in natural aquatic systems may present a potentially serious threat to human health. At present there seems to be no knowledge or consideration of the risk of FLA surviving the treatment and disinfection process or the potential of these protozoa to digest pathogenic bacteria which is able to survive, grow, and exit the FLA creating a further health risk to populations using the effluent from treatment plants (Greub *et al.;* 2003; Fukumoto *et al.,* 2010; Thomas *et al.,* 2010; Ovrutsky *et al.,* 2013; Samhan *et al.*, 2015; Waso, *et al.* 2017).

1.3.7.1 Free living amoebae (FLA)

Free-living amoebae (FLA) are unicellular protozoa that feed on bacteria, fungi and algae (Greub and Raoult, 2004; Thomas *et al.,* 2010; Berry *et al.,* 2010; Delafont *et al.*, 2014; Waso, *et al.,* 2017). They are often recovered from soil, water, and clinical samples (Schmitz-

Esser *et al.,* 2008; Niyyati *et al.*, 2015; Waso, *et al.* 2017). Free-living amoebae exist in two developmental stages: the trophozoite (metabolically active form) and the cyst forms (dormant form).

Depending on the species, trophozoites are 25 - 40 µm in diameter. FLA move by producing numerous needle-like projections (acanthopodia) (Waso, *et al.* 2017). Osmotic regulation is enabled by a contractile vacuole in the cytoplasm. Trophozoites feed on microorganisms and multiplication is achieved by binary fission.

Cysts are 10 - 30 µm in diameter depending on the species, are double walled and often polygonal in shape (Visvesvara *et al.,* 2007; Fouque *et al.*, 2012). Cysts are in a resting stage and have a specific envelope made up of the ectocyst and the endocyst. For their survival, amoebae will encyst under conditions of adverse pH, osmotic pressure, temperature conditions and nutrient depletion and will only excyst when conditions become favourable again (Greub and Raoult, 2003; Visvesvara *et al.,* 2007; Delafont *et al.*, 2014; Waso, *et al.* 2017).

The main function of free-living amoebae (FLA) in nature is as predators of micro-organisms, maintaining soil fertility and cycling nutrients in aquatic food chains (Pagnier *et al.,* 2008; Thomas *et al.,* 2010; Berry *et al.,* 2010; Delafont *et al.*, 2014; Goni *et al.*, 2014; Waso, *et al.* 2017). However, their presence in potable water, hydrotherapy baths, cooling towers, HVAC systems and other sources, may present a potentially serious threat to human health as two genera (*Acanthamoeba* and *Naegleria*) are known human pathogens (Greub *et al.,* 2003; Marciano-Cabral and Cabral, 2007; Ovrutsky *et al.,* 2013; Coskun *et al.,* 2013; Delafont *et al.*, 2014; Waso, *et al.* 2017).

These FLA have been found to be present in dental unit water lines, which can increase the risk of human infection indirectly as they can support the growth of amoeba resistant bacteria (Greub and Raoult 2003, 2004; Coskun *et al.*., 2013). The pathogenic free-living amoebae such as *Acanthamoeba* and *Naegleria* have been recovered from drinking water, cooling towers, swimming pools and hospital water networks (Thomas *et al..,* 2010; Zanella *et al..,* 2012; Coskun *et al.*., 2013; Delafont *et al.*, 2014, Waso, *et al.* 2017; Dobrowsky, Khan and Wesaal, 2017).

FLA make significant contributions to nutrient fluxes (Molmeret *et al.,* 2005) by grazing on bacteria and phytoplankton, but can be reservoirs of human pathogens (Thomas *et al.,* 2010; Coskun *et al.,* 2013).

Acanthamoeba species are one of the most prevalent protozoa species found in the environment (Zanella *et al.,* 2012) and have been isolated from soil, dust, air, natural and treated water, seawater, swimming pools, sewage, sediments, air-conditioning units, domestic tap water, drinking water treatment plants and mammalian cell cultures (Coskun *et al.,* 2013; Dobrowsky *et al.* 2017).

The genus Acanthamoeba contains 9 species such as *A. culbertsoni, A. polyphaga, A. castellanii, A. astronyxis, A. hatchetti, A. rhysodes, A. divionensis, A. lugdunensis, and A. lenticulata* which are implicated in human infection, what makes these organisms such a big risk factor is the fact that Acanthamoeba cysts are resistant to extremes of pH, temperature, disinfection and desiccation. The above-mentioned characteristics account for the presence of the organism in soil, natural and artificial waters, chlorinated swimming pools and the atmosphere (Marciano-Cabral and Cabral, 2007; Ovrutsky *et. al*., 2013; Coskun *et. al*., 2013). However, Acanthamoeba cannot exist in the trophozoite form in environments with low oxygen levels for long periods of time as it is an aerobic organism (Khan, 2006; Lambrecht *et al.*,2015).

Apart from the fact that they can harbour pathogens (Schuster and Visvesvara, 2004; Visvesvara *et al.,* 2007; Pagnier *et al.,* 2009, Lambrecht *et. al*.,2015; Dobrowsky *et.al.,* 2017; Waso *et.al.,* 2017; Inkinen *et.al*., 2019) some species of *Acanthamoeba* are pathogenic to humans. Two distinct diseases can be ascribed to *Acanthamoeba* species: A central nervous system (CNS) infection called Granulomatous Amoebic Encephalitis (GAE) (Abd *et al.,* 2009b; Reddy *et al.*, 2011; Dobrowsky *et.al.,* 2017) and inflammation of the cornea (keratitis) in which they infect the eye (Dini *et al.,* 2000; Khan, 2006; Coskun *et al.,* 2013; Niyyati *et al.*, 2015; Dobrowsky *et.al.,* 2017). *A. castellanii* are most frequently reported as causing keratitis, and *A. culbertsoni* is most frequently reported as causing GAE (Khan, 2006; Coskun *et al.,* 2013; Niyyati *et al.*, 2015; Dobrowsky *et.al.,* 2017). Granulomatous amoebic encephalitis is a chronic disease of immunosuppressed hosts suffering from concurrent diseases such as AIDS (MacLean *et al.,* 2007; Barratt, *et.al.*, 2010) or alcoholism which predispose them to opportunistic infections. Symptoms of granulomatous amoebic encephalitis (GAE) include fever, headache, seizures, meningitis, and visual abnormalities.

Diagnosis is most often made by post-mortem examination of brain tissue (Binesh *et al.,* 2011). *Acanthamoeba* keratitis was first noted in individuals suffering corneal trauma due to injury to the corneal surface that became infected with *Acanthamoeba*. (Khan, 2006; Coskun

et al., 2013). *Acanthamoeba* keratitis causes a potentially blinding effect of the cornea and can lead to permanent blindness. The disease is characterized by intense pain and ring-shaped infiltrates in the corneal stroma (Khan, 2006; Coskun *et al.,* 2013). More commonly, *Acanthamoeba* keratitis occurs in contact lens users when, due to improper maintenance and poor sanitary precautions, like rinsing and storing of lenses in tap water or non-sterile saline solutions, cause *Acanthamoeba* to proliferate in the ophthalmic solutions or in the lens cases and are transferred to the corneal surface when the lens is inserted (Dini *et al.,* 2000, Khan, 2006; Coskun *et al.,* 2013).

The other potentially pathogenic FLA species is *Naegleria* which is found in natural or man-made lakes and thermally polluted freshwater where they can feed upon bacteria and proliferate (Waso, *et.al.,* 2017). This amoeba is a facultative pathogen capable of living many generations without infecting a host and can tolerate temperatures up to 46°C (Schmitz-Esser *et al.,* 2008; Thomas *et al.,* 2010). Primary amoebic meningoencephalitis (PAM) a fulminating, rapidly fatal infection of the central nervous system (CNS) is most often caused by *Naegleria fowleri*, but other species of *Naegleria* with pathogenic potential have been described such as *Naegleria australiensis* and *Naegleria italica* (Schuster, 2002; Thomas and Ashbolt, 2011; Waso, *et.al.* 2017).

1.3.7.2 Amoeba resistant bacteria (ARB)

Some microorganisms have evolved to become resistant to protozoa digestion such that they are able to survive, grow, and exit free-living amoebae after internalization. (Greub and Raoult, 2004; Thomas *et al.,* 2008; Loret and Greub, 2010; Thomas *et al.,* 2010; Delafont *et al.*, 2013, 2014; Goni *et al.*, 2014; Scheid, 2014; Lambrecht *et.al.* 2015; Dobrowsky *et al.* 2017). At least 146 ARB have been identified to date and the list continues to grow (Huws *et al.,* 2006; Steinberg *et al.,* 2007; Thomas *et al.,* 2008; Loret and Greub, 2010; Goni *et al.*, 2014; Scheid, 2014; Hamilton *et.al.,*2019). ARB can infect humans through inhalation of aerosols, direct contact and ingestion of contaminated water or food products (Huws *et al.,* 2008; Abd *et al.,* 2009a; Conza *et al.*, 2013; Bentham and Whiley, 2018; Gebert *et.al.*, 2018). Table 1.1 describes the pathogenicity and common amoebal host and Table 1.2 the lifestyle of the ARB targeted in this study.

FLA play a major role in the spread of pathogenic *Vibrio* species (Abd *et al.,* 2010; Lutz *et al.* 2013; Goni *et al.*, 2014, Hamilton *et.al.,*2019), *Campylobacter jejuni* (Axelsson-Olsson *et al.,* 2005; Bui *et al.*, 2012; Olofsson *et al.,* 2013; Denoncourt *et al.*, 2014; Olofsson *et al.,* 2015;

Hamilton *et.al.,*2019), *Francisella tularensis* (Abd *et al.,* 2003; El-Etr *et al.,* 2009; Thomas *et al.,* 2010; Fouque *et al.*, 2012; Hamilton *et al.,*2019), *Helicobacter pylori* (Winiecka-Krusnell *et al.,* 2002; Gião, *et al.,* 2011; Denoncourt *et al.*, 2014; Hamilton *et.al.,*2019), *Legionella pneumophila* (Zusman *et al.,* 2004; Gião, *et al.,* 2011; Al-Quadan *et al.,* 2012; Denoncourt *et al.*, 2014; Ji *et al.*, 2014; Muchesa *et al.* 2015; Hamilton *et.al.,*2019), *Salmonella typhimurium* (Gaze *et al.,* 2003; Bleasdale *et al.,* 2009; Douesnard-Malo and Daigle, 2011; Bui *et al.*, 2012; Denoncourt *et al.*, 2014, Hamilton *et.al.,*2019) and *Shigella* dysenteriae (Saeed *et al.,* 2009, 2012; Hamilton *et al.,*2019) acting as reservoirs, vectors, and hosts. This was confirmed when Gram negative bacteria of different origin were demonstrated as endosymbionts in *Acanthamoeba* species. Although issues surrounding FLA and ARB are becoming more prominent research shows that the information based on FLA and ARB is limited (Valster *et al.,* 2009; Thomas and Ashbolt, 2011; Hamilton *et.al.,*2019).

In the South African context *Legionella* species, environmental *Mycobacterium* species, particularly members of the *Mycobacterium avium* complex (MAC) and environmental *Chlamydia* species, including *Chlamydophila pneumoniae* and *Parachlamydia acanthamoebae* (Hall's coccus) are of particular importance. The aforementioned ARB poses an increased risk of disease taken that a large section of the South African under- privileged communities who are more likely to be exposed to these ARB is HIV positive and could be immunocompromised (Bartie *et al.,* 1997; Greub *et al.,* 2003; Angenent *et al.,* 2005, Singh and Coogan, 2005; Singh and Matuka, 2013, Muchesa, *et.al.,*2015).

Table 1.1: Selected ARB's their preferred host and pathogenic effects.

ORGANISM DESCRIPTION	Most Common Amoebal Host	PATHOGENIC EFFECTS	References
Escherichia coli 0157	*Acanthamoeba*	Diarrhoea and abdominal cramps	Greub and Raoult, 2004; Thomas *et al.,* 2008; Scheid, 2014
Salmonella species	*Acanthamoeba*	Diarrhoea and abdominal cramps, typhoid fever	Thomas *et al.,* 2008; Thomas *et al.,* 2010; Scheid, 2014
Shigella species	*Acanthamoeba*	Diarrhoea and abdominal cramps	Thomas *et al.,* 2008; Thomas *et al.,* 2010; Scheid, 2014
Vibrio cholerae	*Acanthamoeba, Naegleria fowleri*	Cholera	Greub and Raoult, 2004; Thomas *et al.,* 2008; Scheid, 2014
Legionella species	*Acanthamoeba*	Legionellosis, flu-like disease (Pontiac fever)	Greub and Raoult, 2004; Thomas *et al.,* 2008; Scheid, 2014
Mycobacterium avium complex	*Acanthamoeba*	Respiratory infections (main cause in AIDS patients)	Greub and Raoult, 2004; Thomas *et al.,* 2008; Scheid, 2014
Chlamydia pneumoniae	*Acanthamoeba*	Acute respiratory disease (bronchitis, sinusitis, asthma and pneumonia)	Greub and Raoult, 2004; Thomas *et al.,* 2008; Scheid, 2014

Bichai *et al.* (2008) demonstrated the presence of amoebae infected by bacteria in 22/40 (55%) of the cooling towers they studied. Most of these FLA were infected with ARB other

than *L. pneumophila.* Methycillin-resistant *Staphylococcus aureus* (MRSA) has also been shown to survive within amoebae (Huws *et al.,* 2006, Steinberg *et al.,* 2007; Mella *et al.*, 2016).

Table 1.2: Intra-amoebal lifestyle of selected ARB's

ORGANISM DESCRIPTION	Most Common Amoebal Host	Lifestyle	References
Escherichia coli 0157	*Acanthamoeba*	Intracellular Multiplication	Thomas *et al.,* 2010; Mella *et al.*,2016
Salmonella species	*Acanthamoeba*	Intracellular Multiplication	Thomas *et al.,* 2010; Mella *et al.*,2016
Shigella species	*Acanthamoeba*	Intracellular Multiplication, co-culture without lysis	Thomas *et al.,* 2010; Mella *et al.*,2016
Vibrio cholerae	*Acanthamoeba* , *Naegleria fowleri*	Intracellular Multiplication and Intracyst survival	Thomas *et al.,* 2010; Mella *et al.*,2016
Legionella species	*Acanthamoeba*	Intracellular Multiplication and Intracyst survival	Thomas *et al.,* 2010; Mella *et al.*,2016
Mycobacterium avium complex	*Acanthamoeba*	Intracellular Multiplication and Intracyst survival	Thomas *et al.,* 2010; Mella *et al.*,2016
Chlamydia pneumoniae	*Acanthamoeba*	Intracellular Survival	Thomas *et al.,* 2010; Mella *et al.*,2016

There are three groups of ARB namely: those that multiply and cause lysis in amoebal cell, such as *Legionella* and *Listeria* species, those that multiply within amoebae without causing cell lysis (endosymbionts), such as *Vibrio cholerae* and those that survive within the amoeba without multiplying, such as certain coliforms and *Mycobacteria* (Bichai *et al.,* 2008; Isberg *et al.,* 2009; Siddiqui *et al.,* 2011; Mella *et al.*, 2016). In a country such as South Africa which is an advanced developing country surrounded by less developed countries the transfer of

pathogens is highly likely because of migrant labour and immigration. Furthermore, with the wastewater treatment being under pressure due to expanding population the mere fact that pathogens can survive the normal treatment process as suggested by Bichai *et al.* (2008, 2009) is highly problematic. A further concern is the fact that ARB's ingested in amoebae can exchange genes with other bacteria and the host which could turn previous non-pathogenic bacteria into human pathogens and bacteria that was sensitive to antibiotics into antibiotic resistant bacteria or lastly change the way they grow in such a way that the normal methods of identification would not recognise such bacteria (Thomas *et al.* 2010; Gimenez *et al.,* 2011; Mella *et al.*, 2016).

Many FLA recognize and select their food and different ARB prefer different host FLA, meaning that the abundance of FLA depends on the availability of bacterial food (Valster *et al.,* 2009; Mella *et al.*, 2016). International reviews reported that *Acanthamoeba* species are the most common host of ARB, which is the reason *A. castellanii* was selected as the targeted FLA for this study (Greub and Raoult, 2004 and Bichai *et al.,* 2008, Thomas *et al.,* 2008, 2010; Mella *et al.*, 2016).

1.3.7.2.1 Legionella species

Legionella species are Gram negative, aerobic, and non-spore forming bacilli (Hsu *et al.,* 2011; Mercante and Winchell, 2015) which can multiply intra-cellularly within FLA (Greub and Raoult, 2004; Hsu *et al.,* 2011; Mercante and Winchell, 2015; Dobrowsky, *et.al.*, 2017; Waso *et.al.* 2017). *L. pneumophila* was the first ARB for which the important roles of free-living amoebae as reservoirs, vectors (Mercante and Winchell, 2015; Dobrowsky, *et.al.*, 2017; Waso *et.al.* 2017), and an "evolutionary crib" (organisms uses the phagosomes to exchange genes (Greub and Raoult, 2004; Gimenez *et al.,* 2011; Rubenièa, *et.al.* 2017)) have been identified. Forty-eight different Legionella species and 70 serogroups have been described to date (Percival *et al.,* 2004; Corsaro *et al.,* 2010; Lamoth and Greub, 2010; Ji *et al.*, 2014; Mercante and Winchell, 2015). Literature further shows that all *Legionella* species share the same aquatic environment as *L. pneumophila* (Lamoth and Greub, 2010; Mercante and Winchell, 2015, Dobrowsky, Khan, and Wesaal, 2017).

Apart from the areas where FLA are found *Legionella* species can also be found in fountains, oil/water emulsions used for lubricating lathers, misting devices, decorative fountains and water features, dentistry tools, thermostatic mixing valves, compost and potting soil mixes (Mercante and Winchell, 2015; Bentham and Whiley 2018). The most common mode of

transmission is through inhalation of contaminated airborne droplets (Alli *et al.,* 2011; Hsu *et al.,* 2011; Phin *et al.,* 2012; Ji *et al.*, 2014; Mercante and Winchell, 2015; Bentham and Whiley 2018).

Twelve cases of *Legionnaires* disease were identified at a Johannesburg teaching hospital (South Africa) between 1985 and 1986 (Strebel *et al.,* 1988), although Legionella was cultured from the hot water system of the hospital it could not be directly linked to the patients that acquired the infection and the respirators, they were on were suspected. Only two reports could be found about Legionella in South Africa in the last five years. Singh and Coogan (2005) reported the presence of Legionella and amoeba laden with Legionella in the dental unit waterlines of the Johannesburg Teaching hospitals dental clinic. The municipal water supplying the hospital were implicated in the spread of this pathogen. The second incident was a case study of a receptionist at a dental practice in the Western Cape, were again the water coolant system at the dental practice were implicated in the disease (Chikte *et al.,* 2011). A further two cases have been reported at a paper mill in Mpumalanga province in 2007 (South Africa) (Singh and Matuka, 2013). During a study done in South Africa between 2012 and 2014 by Wolter *et al.* (2016) the problem of masked Legionella infections was highlighted, as most of the identified cases were masked by tuberculosis and other lower respiratory infections and thus no proper treatment was afforded to the patients.

Legionella species grown within amoebae could undergo physiological modifications and become more resistant and more virulent (Alli *et al.,* 2011; Gimenez *et al.,* 2011; Ji *et al.*, 2014; Dobrowsky, *et.al.*, 2017; Bentham and Whiley 2018). Numerous studies concerning the relationship between FLA and *Legionella* have confirmed that FLA are necessary for *Legionella* multiplication in water biofilms, although the bacteria may survive in a latent state in biofilms without amoebae (Greub and Raoult, 2004, Gimenez *et al.,* 2011; Mercante and Winchell, 2015; Dobrowsky, *et.al.*, 2017).

Legionella-like amoebal pathogens (LLAP) are bacilli that to date have not been found to grow on laboratory media, but they infect and multiply in the cytoplasm of amoebae. LLAP were initially described by Rowbotham in 1983 and may be of considerable importance because they have been shown to be pathogenic since they cause pneumonia and induce a serological response in infected patients (Moliner *et al.,* 2010). Some of the LLAP species have since been incorporated into the genus I based on genetic similarities even though LLAP were originally thought to be a separate genus (Moliner *et al.,* 2010). It was suggested by Greub and Raoult (2004) that, humans are infected not by inhaling free Legionella (Hsu *et al.,*

2011) but by inhaling a vesicle or an amoebae filled with Legionella organisms (Bozzaro and Eichinger, 2011; Mercante and Winchell, 2015; Dobrowsky, *et.al.*, 2017) this could lead to both *legionnaires* disease as well as Pontiac fever depending on the *legionella* bacteria enhaled.

Eighty percent of all cases of human *legionellosis* are caused by *L. pneumophila*, with approximately half of the 48 *Legionella* species associated with the remaining disease cases (Yanez *et al.,* 2005; Lamoth and Greub, 2010). Water is the major reservoir for Legionellae (Loret and Greub 2010; Dobrowsky, *et.al.*, 2017) and water systems as reservoirs for agents associated with respiratory tract infections were brought into sharp focus when *L. pneumophila* was identified as the cause of an outbreak of pneumonia in 1976 during the American Legion convention at the Bellevue-Stratford Hotel in Philadelphia. During that outbreak, 221 individuals became ill, and there were 34 deaths. After *legionellosis* was isolated and characterized in 1977 it became apparent that between 2 to 7% of community-acquired pneumonia (CAP) affecting both immune competent and immune-compromised hosts (Janda, 2010) can be attributed to *Legionella* species.

Legionella species infect patients by either inhalation of contaminated aerosols or by aspiration of contaminated water (Mercante and Winchell, 2015, Bentham and Whiley, 2018; Gebert *et al.*.,2018). The infection progresses to the lungs, which leads to *legionellosis* (Yanez *et al.,* 2005) which consists of two distinct clinical syndromes, Legionnaires' disease, and Pontiac fever. Legionnaires' disease is characterized by pneumonia while Pontiac fever is a self-limited, non-pneumonic, respiratory illness (Phin *et al.,* 2012). In the pneumonic form of *legionellosis*, the bacteria multiply intracellularly in the alveolar macrophages of an infected (often immune-compromised) host. The ability to multiply intracellularly is considered the primary virulence factor of *Legionella* species. Legionnaires' disease present with similar symptoms than other forms of pneumonia namely high fever, chills and cough, with muscle aches and headaches reported in some patients and can sometimes be masked by these or misdiagnosed as the more common causes of pneumonia (Wolter *et al.*, 2016). It takes 2 to 14 days after being exposed to Legionellae for the onset of these symptoms (Potts, *et al.,* 2013).

1.3.7.2.2 Escherichia coli

Escherichia coli were considered for many years to be a commensal organism of the large intestine. There are six different pathogenic strains of *Escherichia coli* namely, the

enterotoxigenic, enteropathogenic, enterohaemorrhagic, enteroinvasive, enteroaggregative and diffusely adherent strains which can lead to human diseases particularly diarrhoea through different mechanisms (Todar, 2007; Galván-Moroyoqui *et al.,* 2008; Rojas-Lopez *et al.* 2018). The presence of *Escherichia coli* is used as a useful marker of feacal water contamination (Todar, 2007; Galván-Moroyoqui *et al.,* 2008; Farnleitner *et al.,* 2010, Senkbeil *et al.* 2019), however the absence of the organism does not mean that the water is safe, as FLA can also harbour and facilitate the intracellular growth of Gram-negative bacteria such as *Escherichia coli*, (Thomas *et al.,* 2010; Dobrowsky, *et.al.*, 2017) and make them undetectable using the standard water quality testing. Research has shown that Gram negative bacteria such as *Escherichia coli* are abundantly found in water and are therefore available as a major source of food for amoebae more so than other bacteria and is often used to isolate trophozoites. (Alsam *et al.,* 2006; Thomas *et al.,* 2010, Senkbeil *et al.* 2019).

Alsam *et al.* (2006) reported clear differences between invasive and non-invasive *Escherichia coli* strains pertaining to their ability to survive intra-cellularly in *A. castellanii* although the researchers could not shed light on the mechanisms the organisms uses to survive (Mohamed, *et.al.* 2016; Lambrecht *et.al.* 2015). It was however postulated that the same mechanisms is used as that used by *Legionella* species in the amoebal intracellular survival.

The clinical presentation of diarrhoea varies according to the strain causing the infection. Some of the *Escherichia coli* strains may lead to neonatal meningitis wherein the meninges (tissue that covers the brain and spinal cord) are inflamed. Other strains may also cause urinary tract infection which manifest by burning pain in the pelvic area (Harrison, 2011).

Table 1.3 Reported cases of *Escherichia coli* in South Africa 2009-2011**.**

E coli isolates	2009	2010	2011
South Africa	520	534	234
Gauteng	304	339	123
0 – 4 year Old	220	460	188

Adapted from Keddy (2010,2011,2012)

Table 1.4 Different strains isolated from disease cases in Gauteng between 2009-2011

Year	DAEC	EAggEC	STEC/ EHEC	EIEC	EPEC	ETEC
2009	77	75	10	4	342	41
2010	90	58	8	7	358	13
2011	33	25	2	5	167	2

DAEC: Diffusely-adherent *Escherichia coli*; **EAggEC:** entroaggregative *Escherichia coli*; **STEC/EHEC:** Shiga-toxigenic *Escherichia coli* or entro-haemorrhagic *Escherichia coli*; **EIEC:** enteroinvasive *Escherichia coli;* **EPEC:** enteropathogenic *Escherichia coli*; **ETEC:** enterotoxigenic *Escherichia coli.*

Data in tables 1.3 and 1.4 was obtained from disease surveillance done in South Africa by the National Health Laboratory Services (NHLS) and the National Institute for Communicable Diseases and yearly reported in the Communicable Diseases surveillance bulletin (Keddy, 2010, 2011 and 2012). The surveillance data revealed that, the Gauteng province had the highest incidence of diarrhoea caused by *Escherichia coli* in South Africa. Okeke (2009) noted that the EPEC and the EAEC groups was the most common cause of diarrhoea in HIV positive individuals. Furthermore, according to this data (Table 1.3,1.4) the most prevalent strain found in surveyed cases of diarrhoea in Gauteng was the enteropathogenic E Coli species which included the O55, O111, O119 and the O127 serotypes most often over the 3 years reported (Keddy, 2010, 2011 and 2012). However other more virulent strains of EPEC such as O157 has also been isolated in 2010 and 2011. According to Keddy (2011) this serotype of *E Coli* are found in both the EHEC and the EPEC group. This specific serotype was involved in the 1990 sporadic Haemorrhagic Uremic Syndrome (HUS) in South Africa, which was the first report of this serotype in Africa, and three years later the outbreak in Swaziland which claimed 2000 lives (Okeke, 2009). However, the *Escherichia coli* identified in these cases were entero-haemorrhagic *Escherichia coli* (Okeke, 2009). Even though the prevalence of EHEC is low in this province it seems that some form of gene swapping is taking place with both EHEC and EPEC presenting with the serotype O157. This gene swapping taking place can according to Gimenez *et al.,* (2011) be due to these organisms being in close contact in the phagosomes of FLA's (Ashbolt, 2015). This can be a greater

concern to public health than just the mere survival of these organisms through the treatment process.

1.3.7.2.3 Salmonella

The genus *Salmonella* is part of the family Enterobacteriaceae which consists of over 2,500 different types of bacteria. These types are distinctly identifiable by the specific protein coating they display. *Salmonella* strains are flagellated, Gram-negative bacilli (Perilla, 2003). One of the waterborne diseases commonly caused by *Salmonella* is typhoid fever. Typhoid fever is common in developing countries (Iperepolu *et al.,* 2008; Kariuki, 2008; Kabwama, *et.al.* 2017) and has an estimated 21 million cases with over 200 000 deaths annually worldwide (Crump *et al.,* 2004; Dewan *et al.*, 2013; Kabwama, *et.al.,* 2017). Numerous studies reported typhoid fever to be endemic in developing countries of the Indian subcontinent, Southeast Asia and in Africa (Iperepolu *et al.,* 2008; Kothari *et al.,* 2008; Dewan *et al.*, 2013; Kabwama, *et.al.* 2017) mainly due to poor water treatment infrastructures and sanitation facilities (Kariuki, 2008; Oguntoke *et al.,* 2009; Dewan *et al.*, 2013; Kabwama, *et.al.* 2017). In South Africa, an outbreak of typhoid fever was reported in Mpumalanga province in 1993, where more than 2000 cases were recorded**.** Again, in September 2005, 380 cases of diarrhoea, suspected cases of typhoid fever and nine confirmed cases occurred in the same area of Mpumalanga province (Keddy *et al.,* 2011; Mpenyana-Monyatsi *et al.*, 2012).

Table 1.5 Reported cases of *Salmonella* Typhi in South Africa from the years 2009-2011.

Samonella Typhi isolates	2009	2010	2011
South Africa	8/58	18/58	9/63*
Gauteng	1/26	4/25	3/17
0 – 4 year Old	7	10	8

*Non-Invasive/Invasive *Salmonella* Typhi isolates. Adapted from Keddy (2010, 2011, 2012)

Table 1.6
Reported cases of *Salmonella* Non-Typhiod in South Africa from the years 2009-2011.

Samonella Non-Typhiodal isolates	2009	2010	2011
South Africa	1792/763	1570/674	1441/608*
Gauteng	849/396	706/381	575/307
0 – 4 year Old	711/215	613/184	560/165

Non-Invasive/Invasive *Samonella* non-Typhiodal isolates. Adapted from Keddy (2010, 2011, 2012)

Table 1.7 Different serotypes of *Salmonella* Non-Typhiod in Gauteng from the years 2009-2011

Year	Dublin	Enteritidis	Heidelberg	Newport	Infantis	Isangi	Typhimurium
2009	19	223	0	0	182	28	326
2010	337	0	15	0	27	18	295
2011	339	0	12	16	0	12	156

As observed from the above tables (1.5; 1.6; 1.7), as far as diarrhoeal cases caused by *Salmonella typhi* and *non-typhi* in South Africa is concerned, the Gauteng province was the worst affected. As with other diarrhoeal diseases, diarrhoeal disease caused by salmonella. affected 0 - 4-year-old children the worst (Keddy, 2010, 2011, 2012, Keddy *et.al.* 2018).

Many authors have described the survival of *S. typhimurium* and other serovars inside amoeba (Adiba *et al.,* 2010; Bozzaro and Eichinger, 2011; Douesnard-Malo and Daigle, 2011; Riquelme *et al.* 2016) under different conditions including chlorination (Brandl *et al.,* 2005; Bridier *et al.,* 2011**;** Denoncourt *et al.,* 2014; Mohamed, *et.al.* 2016**)**. In an experiment conducted by King *et al.,* (1988) it was found that these intracellular bacteria are up to 50-fold more resistant to high levels of free chlorine as was confirmed by later studies (Cervero-Arago *et al.*, 2015; Ashbolt 2015).

1.3.7.2.4 Shigella species

Shigella species are highly adapted human pathogens that can cause shigellosis (an abdominal pains or even dysentery characterized by diarrhoea, fever, vomiting tenesmus and stools containing blood, pus, or mucus) (Warren *et al.,* 2006; Addis and Sisay 2015). The World Health Organization has reported that *Shigella* diseases are widespread with estimates of 164.7 million cases per year in total, with 163.2 million cases occurring in developing countries (Abelman *et.al.* 2019; Koestler *et al.* 2019) and 1.5 million cases in industrialized countries, with more than 60% of these cases occurring in children under 5 years old (Bardhan *et al.,* 2010; Mbuthia *et.al.* 2018; Vubil *et.al.* 2018). The mortality rate (2007-2012) in developing countries was estimated at 1.1 million deaths per year. Most episodes (69%) and death (61%) occurred in children under 5 years old. (Mbuthia *et.al.* 2018; Vubil *et.al.* 2018) These figures have been decreasing in the last decade with the reported disease burden in 2009 estimated at 150 million episodes with 1 million deaths in developing countries (Kinge and Mbewe, 2010; Vubil *et.al.* 2018) and in 2012, Koh *et al.* reported the disease burden of 90 million and 108000 deaths. The estimates in 2009 for South Africa was estimated on 1812 cases (Keddy,2010), 2010 it was 1753 (Keddy,2011), 2011 it was estimated on 1685 (Keddy, 2012), at this time the 2012 final figures were not out but the 2013 figure to June has already been estimated at 875 cases reported for the year. In a recent study conducted by researchers from university of Cape Town (UCT)and the National Health Laboratory Services (NHLS) (2015-2016) in Cape Town *shigella flexineri* was the second most prevalent pathogen, although between 2003 -2009 it was the most common cause of shigellosis diagnosed in South Africa (Kalule *et al.* 2019).

Table 1.8 Reported cases of *Shigella* in South Africa between the year 2009 and 2011.

Shigella isolates	2009	2010	2011
South Africa	1744/68	1704/49	1618/67*
Gauteng	664/21	692/19	619/26
0 – 4 year Old	810/27	820/16	783/33

*Non-Invasive/Invasive *Shigela* isolates. Adapted from Keddy (2010,2011,2012)

Table 1.9 Different strains isolated from disease cases in Gauteng between the year 2009 and 2011

Year	*S. Dysenteria type 1*	*S.Flexneri type 1b*	*S.Flexneri type 2a*	*S.Flexneri type 3a*	*S.Flexneri type 6*	*S.Sonnei phase I/II*
2009	1	158	57		70	199
2010	0	0	154	60	60	237
2011	0	0	164	56	67	193

Although we notice an overall drop in the yearly reported cases, and we notice that no fatalities were reported in these years it is still alarming that over a thousand cases of this disease are reported yearly with the largest proportion in Gauteng and the most affected age group is children under 5 years of age. (See table 1.8) (Keddy 2010, 2011, 2012).

Although none of the environmental studies has reported *Shigella* as one of the ARB, Jeong *et al.* (2007) and Saeed *et al.* (2009, 2012) showed with laboratory experiments that *S. sonnei* and *S. dysenteriae* are not only internalised by FLA, but may also survive within the amoeba, making it a candidate for a very pathogenic ARB (Rubenìòa, *et.al.* 2017). Furthermore research has shown that *S. sonnei* internalised in amoeba during chlorination and that the organism resisted free chlorine disinfection by an order of 30-120-fold (Bichai *et al.,* 2008; Cervero-Arago *et al.*, 2015; Rubenìòa, *et al.* 2017). Although *Shigella dysentteria* is rare in Gauteng, *Shigella sonnei* is quite commonly isolated in disease cases in Gauteng and recently *Shigella flexineri* were isolated from the water and sediment samples from the Apies river (Ekwanzala *et al.* 2017) (see Table 1.9) (Keddy, 2010, 2011, 2012). In the light of this information and the severe disease it causes, it is necessary to add this organism to the list to be analysed from wastewater samples.

1.3.7.2.5 Vibrio species

Vibrio cholerae is a motile Gram negative curved-rod bacillus. The genus vibrio consists of 71 species of which twelve species are presently known to cause or to be associated with human infections: *V. alginolyticus, V. carchariae, V. cholerae, V. cincinnatiensis, V. damsela,*

V. fluvialis, V. furnissii, V. hollisae, V. metschnikovii, V. mimicus, V. parahaemolyticus and *V. vulnificus*. More recently, the genus *Vibrio* has been considered to consist of 100 species of which 14 are considered to be pathogenic (Morris, 2013; Romalde *et al.*, 2014). The most serious human pathogens were found to be *V. cholerae, V. parahaemolyticus*, and *V. vulnificus* (Thompson *et al.,* 2004; Romalde *et al.*, 2014; Kokashvili et. al. 2015).

Free-living amoebae act as reservoirs for *V. cholerae* in natural waters in most parts of the world where, there is no evident association with clinical cases of cholera. This non-detectable environmental persistence of this organism could be a potential risk for possible cholera infections if the amoebae are killed or the cholera bacteria are released (Lutz *et al.,* 2013**)**. *Acanthamoeba* species and *Vibrio cholerae* are observed in the aquatic environments in cholera endemic areas as well as in drinking water of these areas (Backer, 2002; Greub and Raoult, 2004; Lambrecht *et.al.* 2015; Shanan *et al.*, 2016; Waso, et.al. 2017). Studies reported that *A. polyphaga* enhanced the survival of *V. cholerae* for 2 weeks while the number of the bacteria in absence of amoebae decreased to no detectable level in few days (Sandström *et al.,* 2010). Various studies have shown that *A. castellanii* can host *V. cholerae O1* and *O139* serogroups which are causative agents of cholera (Abd *et al.,* 2005; Abd *et al.,* 2007; Faruque and Nair, 2008; Abd *et al.,* 2009a; Sandström *et al.,* 2010; Shanan *et al.*, 2016).

Cholera is the common pandemic disease, especially in Africa (WHO, 2011) where approximately 2.2 million induvial die because of basic hygiene related diseases, like diarrhoea, every year (WHO, 2002).

In South Africa, during 2000/2001 a cholera epidemic spread through the Eastern and North Eastern parts of KwaZulu Natal. During that time, *Vibrio cholerae El Tor* serotype *Ogawa* was isolated that brought about 82,275 cases to cholera treatment centres (hospitals, clinics, and rehydration centres) and caused 171 deaths (Mari *et al.,* 2012). In 2009, five hundred and sixty-six cases of *V cholera* O1 El Tor Ogawa were reported with the largest concentration in the Limpopo province (Keddy 2010). Arguably, only a single case of this disease was reported in 2010 and 2011 (Keddy 2011, 2012). Unlike *Shigella* the distribution of the disease was not focused in the very young (Keddy 2010).

The troubling question Kùstner and Du Plessis highlighted in their 1991 study of the 1980-1987 cholera outbreaks was how *Vibrio cholerae* survived during non-epidemic periods. Bateman, (2009) speculated that the possible survival strategy could be infestation of biofilms

and since amoeba are often associated with biofilm this could also be a reservoir of these organisms (Loret and Greub, 2010; Shanan *et al.*, 2016). This question could be answered in this study as free-living amoebae could be a plausible reservoir of these and other pathogenic organisms.

1.3.7.2.6 Mycobacterium species

Mycobacteria are aerobic, with a waxy outer coat and non-motile, acid-fast bacilli (except for the species *Mycobacterium marinum,* which has been shown to be motile within macrophages) (Ryan and Ray, 2004; Bozzaro and Eichinger, 2011; Bekale *et.al.* 2018).

Jadin (1975) and again Krishna-Prasad and Gupta in 1978 were the first to report that *Mycobacterium* species survive in FLA, but at the time they could not demonstrate multiplication or lysis. *M. leprae* is generally believed to be the first species in the family of *Mycobacteriaceae* to survive in FLA with studies dating back three decades (Greub and Raoult, 2004; Snelling *et al.,* 2006; Thomas and McDonnell, 2007; Wheat *et al.*, 2014; Cardenal-Muñoz *et al.* 2018) however subsequent, in vitro experiments showed that *M. avium, M. marinum, M. ulcerans, M. simiae,* and *M. habane* could enter free-living amoebae and that *M. smegmatis, M. fortuitum*, and *M. phlei* could be found in large numbers in the amoebae, eventually leading to lysis of the FLA (Thomas and McDonnell, 2007; Eddyani *et al.,* 2008; Medie *et al.*, 2011; Lamrabet *et al.,* 2012; Mella *et al.*, 2016; Cardenal-Muñoz *et al.* 2018).

Non-tuberculosis mycobacteria (NTM) are an example of an ARB showing behaviour similar to that of *Legionella* species, at least in drinking water systems (Falkinham, Pruden, and Edwards 2015). A decrease in *Mycobacterium* concentrations along the treatment lines were observed at two wastewater treatment plants, from raw to ozonated water, an increase after granular activated carbon (GAC) filtration, due to filter colonization, and again a decrease after chlorination (Wang *et al.,* 2013). In 72% of tested samples obtained from the drinking water distribution system of Paris supplied by these two plants NTM were present, with potentially pathogenic mycobacteria accounting for 16% of all positive samples, this has also been the case in Australia and the United States of America (Loret and Greub, 2010; Delafont *et al.*, 2014; Falkinham, Pruden, and Edwards 2015; Molino *et al.* 2019).

Mycobacterium avium complex (MAC) is a further subgroup of genetically related bacteria belonging to the genus *Mycobacterium,* which includes *M. avium and M. intracellulare*. Both these organisms are known to cause opportunistic infections that may result in a severe illness such pulmonary MAC disease. Pulmonary MAC disease is a nontuberculosis mycobacterial lung disease which affects people with advanced AIDS and individuals that are immuno-compromised, and recently reported, people that are otherwise healthy (Chern *et.al.* 2015; Molino *et al.* 2019). Reported cases of NTM infections has showed alarming increases in developed countries, but no information of developing countries is available due to these not falling under the notifiable diseases (Molino *et al.* 2019). Furthermore, the risk of disseminated MAC lung disease is directly related to the severity of immunosuppression (Snelling *et al.,* 2006; Perez-Martinez *et al.*, 2013; van der Wielen and van der Kooij, 2013, Falkinham, Pruden, and Edwards 2015). A pulmonary nontuberculous mycobacterial infection can take one of two forms, the classical form, which is radiographically indistinguishable from tuberculosis, and non-classical (atypical) form.

The strain of the *M. avium complex* is a natural endosymbiont of *Acanthamoeba* (Yu *et al.,* 2007; Ovrutsky *et al.*, 2013). This were observed in a hospital water network study, in which 46.7% of samples from which amoebae were isolated mycobacterial growth were observed as well (Thomas *et al.,* 2006; Delefont *et al.*, 2014; Falkinham, Pruden, and Edwards, 2015).

The fact that amoebae could represent a widespread reservoir for mycobacteria, was confirmed by the association between the presence of amoebae and mycobacteria in man-made aquatic environments (Thomas *et al.,* 2008; Delafont *et al.*, 2014; Falkinham, Pruden, and Edwards 2015). Moreover *M. avium* grown within amoebae were more virulent than those grown in broth medium. This was demonstrated when *M. avium* first, grown in amoebae resulted in enhanced entry into amoebae after sub-culturing, on intestinal epithelial cell line and macrophages. The enhanced virulence was further proven when *M. avium* grown in amoebae showed enhanced ability to colonize the intestine of a mouse model and could be replicated in the liver and the spleen. These studies further demonstrated that *M. avium* was able to survive within cyst walls of *Acanthamoeba* (Snelling *et al.,* 2006; Ovrutsky *et al.*, 2013; Samba-Louaka *et al.* 2018). Moreover, amoebae provided more protection for *M. avium* against antimicrobials usually prescribed as prophylaxis for *M. avium* disease in AIDS patients, including drugs such as rifabutin, clarithromycin, and azithromycin, than did macrophages (Snelling *et al.,* 2006; Kicka *et al.*, 2014).

In 2002, a study of the drinking water in Pretoria, revealed the presence of NTM in biofilms collected from this water distribution system (September *et al.,* 2004). Furthermore, a recent study on the causes of death amongst HIV patients on ARV in Soweto (Gauteng, South Africa) *Mycobacterium avium* Complex were the third leading cause of death amongst these patients (14%) (Karstaedt, 2012). It is for these reasons and the fact that MAC can survive and multiply in FLA that this organism was added to the list of tested organisms.

1.3.7.2.7 Chlamydophila pneumoniae

Chlamydophila pneumoniae may escape detection by the immune system by becoming dormant and residing inside cells in a non-replicating form that may persist for long periods of time (Monack *et al.,* 2004; Potroz and Cho, 2015; Brown *et al.* 2016). It comprises important pathogens of vertebrates, and is a symbiont of FLA. Other obligate intracellular bacterial parasites of *Acanthamoeba*, related to *Chlamydia species*, have been found in amoeba isolated from the nasal mucosa of humans (Greub *et al.,* 2004; Yamane *et al.*, 2015; Brown *et al.* 2016), making amoeba a possible environmental vector for the *Chlamydiaceae*, that are able to replicate within *Acanthamoeba* (Greub and Raoult., 2004; Snelling *et al.,* 2006; Omsland *et al.*, 2014; Brown *et al.* 2016).

Chlamydophila pneumoniae may survive within *Acanthamoeba castelanii* but unlike *Parachlamydiaceae* and *Simkaniaceae*, does not replicate within this species of amoeba. *Chlamydia species* has recently been linked to pneumonia in traumatised patients (Friedman *et al.* 2006; Brown *et al.* 2016). Therefore the role of free-living amoebae as a vector for this agent of lung infections has been proven **(**Greub, 2009). Further additional species of *Chlamydiaceae* that is resistant to destruction by free-living amoebae has been identified by Greub (2009) (Croxatto *et al.*, 2013; Brown *et al.* 2016). Currently it is hypothesized that some intracellular pathogens such as *Chlamydiales* and *Rickettsiales* may have shared a common ancestral host species, such as free-living amoebae, in which they exchanged genes more than one billion years ago (Greub *et al.*, 2004; Greub, 2009; Le *et al.,* 2012; Deschamps, 2014; Brown *et al.,* 2016)

In South Africa, the only studies published regarding this pathogen is in relation to its role in Atypical pneumonia. (Dlamini and Mendelson, 2012; Boyles *et al.* 2017).

1.3.8 Potential risks associated with ignoring FLA and ARB interaction in wastewater treatment and monitoring

Apart from the fact that, the impact of FLA's were ignored up to now as part of the monitoring of the quality of recycled water and that it's ability to harbour and spread pathogenic ARB were recently discovered the fact that it can survive conventional treatment protocols is of grave concern.

The wastewater treatment industry heavily relies on the use of biocides such as Chlorine, Chlorine dioxide and Mono chloramine (Mogoa *et al.,* 2011) to ensure water of high quality. However, amoebae in cyst form (and hence intracellular bacteria) are resistant to these biocides and other water treatment methods and can survive in the cyst form for over 24 years (Coulon *et al.,* 2010; Lambrecht *et.al.,* 2015). This resistance to pH, temperature and antibiotics is provided by the cellulose that is part of the cyst wall and provided a physical barrier to these adverse environmental conditions. Thus, often causes reseeding of water distribution systems with bacteria shortly after treatment, which has enormous economic implications for industries that use high volumes of re-circulating water (Jjemba *et al.,* 2010: Marciano-Cabral *et al.,* 2010; Goñi *et al.,* 2014; Lambrecht *et.al.,* 2015).

Apart from the fact that the FLA can cause reseeding the time the different pathogens spends in the amoeba exchange of genetic material can generate either new pathogens or more virulent strains of bacteria due to exchange of virulence genes and antibiotic resistance genes (Rubenìoà, *et.al.,* 2017). Furthermore, the bacteria can gain genes from the amoeba and vice versa. Galvan-Moroyoqui *et al.* (2008) revealed that amoeba that has ingested *E coli* and *Shigella* caused more damage to monolayers of endothelial cells than those that did not ingest these bacteria. Moliner *et al.* (2010) showed that the bacterial genome of bacteria that divide and grow in amoeba is larger than the free-living bacteria of the same species indicating that there is some exchange of genomic material inside the amoeba phagosome as was suggested by Greub *et al.* in 2004 when he called the amoeba the "evolutionary crib" (Kebbi-Beghdadi and Greub, 2014**).** This was later confirmed by other authors (Gimenez *et al.,* 2011; Rubenìoà, *et.al.* 2017).

Another worrying trait is the fact that the amoeba can be used as the proverbial Trojan Horse (Greub *et al.,* 2004; Denoncourt *et al.,* 2014; Rubenìoà, *et.al.* 2017) to transport the pathogenic bacteria past the first line of defense which is the epithelial barrier. Furthermore in 1994 Cirrillo *et al.* (1997) was the first to show that *Legionella pneumophilla* grown in amoeba were 100-fold more invasive in human epithelial cells and 10-fold in human

macrophages. He followed this discovery in 1997 and showed that *M. avium* that were cultured in amoeba were not only more invasive but also more virulent than those grown on normal agar and later other authors showed similar results with *Salmonella* and other bacteria (Cirrillo *et al.,* 1997; Greub *et al.,* 2004; Gryseels *et al.,* 2012; Khattak *et al.*, 2012; Mella *et al.*, 2016; Gomes *et.al.,* 2018).

Despite the fact that the bacteria can be transported into the human host, amoeba resistant bacteria are released from FLA through vesicles (Bichau *et al.,* 2008; Lau and Asbolt, 2009; Loret and Greub, 2010; Thomas *et al.,* 2010; Rubenìoa, *et.al.,* 2017; Gomes *et.al.* 2018). This may increase the risk of infection considerably due to the aerosolization of these vesicles as evidenced by their ability to be spread to vulnerable persons through inhalation of aerosols, direct contact, and ingestion (Khan *et al.,* 2013). Aerosolized water is probably one of the predominant vehicles for transmission of ARB. Aerosolized water is produced by appliances such as air-conditioning system, showers, clinical respiration devices, and whirlpool baths. Free-living amoebae may also increase the transmission of ARB by producing vesicles of respirable size, each filled with viable bacteria (Greub *et al.,* 2004; Denoncourt *et al.,* 2014; Gebert *et al.*, 2018). Aerosolisation of these vesicles increases the risk of infection to humans since ARB growth in amoebae mimic those within macrophages (Denoncourt *et al.,* 2014; Gebert *et al.*, 2018).

It is clear that the threat of FLA's reaches much further than just harbouring pathogenic bacteria and therefore much more attention should be paid to the elimination of these organisms during the water treatment processes than is currently the case.

1.4 Conclusion

In our quest for securing a high quality of water free of microorganisms that can cause harm to consumers and given the fact that the presence of free living amoebae, which may harbour amoebae resistant bacteria, in wastewater raises serious concerns in the water industry. Due to the role free living amoebae play in spreading pathogenic micro-organisms through water systems, we have attempted, through this study, to provide information on various issues regarding their presence throughout a wastewater treatment plant during four seasons. Detailed information is being provided in the following chapters.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Study Design

This is a descriptive study investigating the survival of FLA and ARB in wastewater during the normal treatment protocols used in wastewater treatment. The study focused on the effects of season, pH and water temperature in the survival of these organisms.

2.2 Study Site

This study was conducted at a wastewater treatment plant in Johannesburg, South Africa.

The selected plant treats sewage collected from the southern suburbs and industrial zones of Johannesburg and Soweto East. The treatment plant was first commissioned between 1984 and 1985. The plant consists of a head of works including screening and de-gritting, diffused air, thickeners for raw sludge, thickeners for waste activated sludge bioreactors incorporating the three-stage Phoredox process configuration, final clarification, calcium hypochlorite disinfection and maturation ponds. Sludge is not treated at this plant but pumped to another plant for dehydration and disposal.

The treatment processes were divided into three stages namely primary treatment (two processes, BOS (Bioreactor Outfall Sewer) and BRF (Bioreactor Feed)), secondary treatment (three treatment processes) and tertiary treatment (three processes)

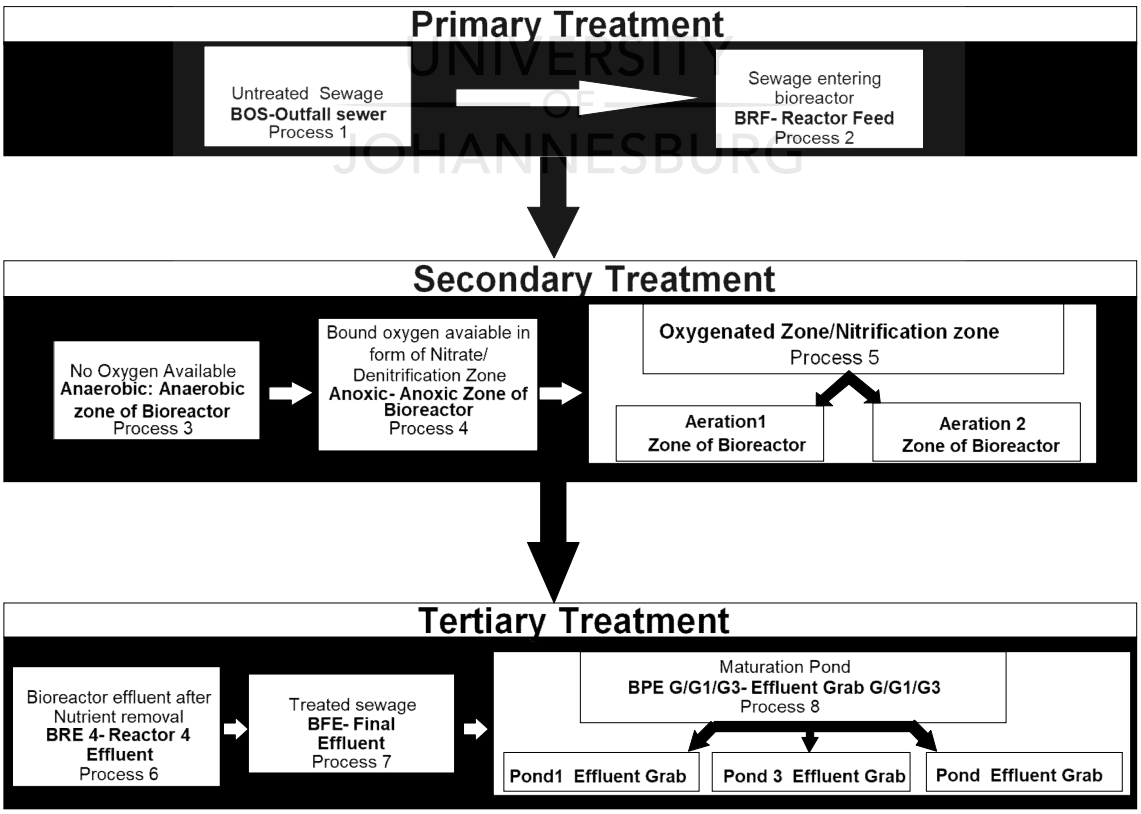


Figure2.1 Treatment Process flow

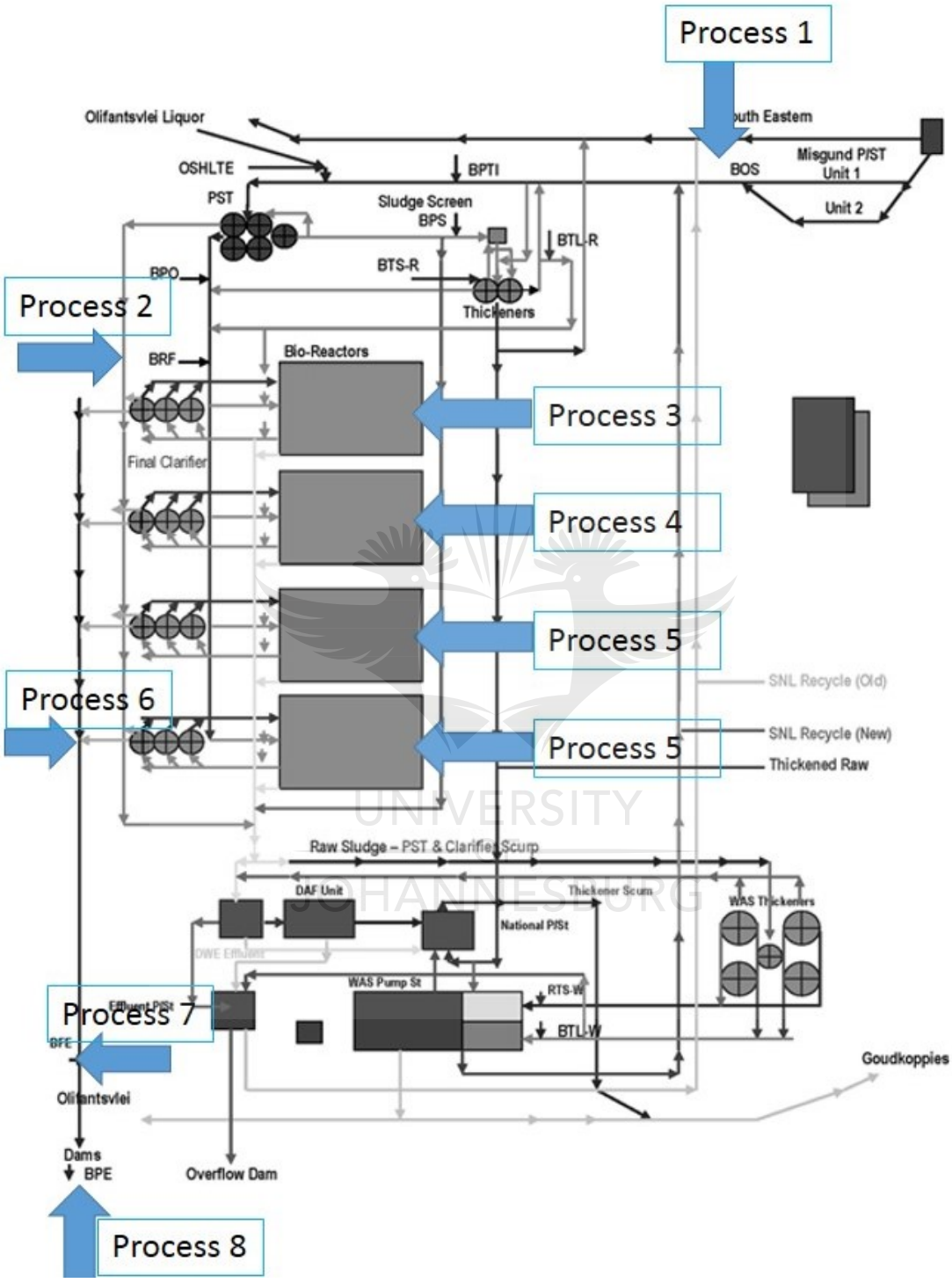


Figure 2.2 Schematic representation of selected wastewater treatment plant (refer to figure 2.1 for simplified process flow chart). (Diagram courtesy of Rand Water)

2.3 Sampling Strategy

The sampling procedure was developed in collaboration with the treatment plant management to coincide with the normal sampling routine of the plant quality control program. Representative samples were collected from each treatment process and if the process consisted of more than one sub process the sampling was split accordingly. The dates and times for sampling were prescribed by the plant management. Samples were collected over a one-year period to test for seasonal variation and the influence of water temperature on amoebae growth. We used a sample size of 500 ml as this is the minimum sample size recommended to obtain sufficient bacteria numbers for analysis. A range of forty-one and forty- four samples were collected per season and eleven to forty-five samples per treatment process. Thirty-two and forty-five samples were collected from Process 5 and Process 8 respectively as these processes each had two to three sampling points. There were two sampling points in Process 5 and three in Process 8 which was essential to obtain a representative picture of the process. The treatment processes are illustrated in Figure 2.1 and will be referred to in the text by the descriptions found in this schematic flow diagram.

Table 2.1 Sample distribution over season and treatment process

Process		SPn	N	Season			
				Autumn	Winter	Spring	Summer
Primary n=36	**1**	**1**	16	4	4	4	4
	2	**1**	20	5	5	4	6
Secondary n=64	**3**	**1**	16	4	4	4	4
	4	**1**	16	4	4	4	4
	5	**2**	32	8	8	8	8
Tertiary n=72	**6**	**1**	16	4	4	4	4
	7	**1**	11	3	2	4	2
	8	**3**	45	9	12	12	12
Total			**172**	**41**	**43**	**44**	**44**

SPn: number of sample points

Figures 2.2, and 2.3 and Table 2.2 show the different sampling sites where water samples were collected. A total of 172 samples were collected in 2010 during autumn (March, April, May), winter (June, July, August), spring (September, October, November) and summer (December, January, February) as summarised in Table 2.1.

Table 2.2: Sample codes and locations

Abbreviation	Process	Meaning	Sample source
BOS	1	Outfall Sewer	Untreated Sewage coming into the works
BRF	2	Reactor Feed	Sewage entering the Bioreactor- untreated
Anaerobic	3	Anaerobic zone of a Bioreactor	No oxygen available
Anoxic	4	Anoxic zone of a Bioreactor	Bound oxygen available in form of nitrate/denitrification zone
Aeration 1	5	Aeration 1 zone of a Bioreactor	Oxygenated zone/Nitrification zone
Aeration 2	5	Aeration 2 zone of a Bioreactor	Oxygenated zone/Nitrification zone
BRE 4	6	Reactor 4 Effluent	Treated effluent from bioreactor No. 4 after nutrient removal
BFE	7	Final Effluent	Combined treated effluent from one bioreactor after nutrient removal
BPE G1	8	Pond 1 Effluent Grab	Maturation Pond No. 1 Effluent- treated
BPE G3	8	Pond 3 Effluent Grab	Maturation Pond No. 3 Effluent- treated
BPE G	8	Pond Effluent Grab	Maturation Pond Effluent – treated

Refer to Figures 2.1 and 2.3 for diagrammatic and flowchart representation of the locations)

2.4 MATERIALS AND EQUIPMENT

All equipment and material for this project were provided by the laboratories of the National Institute for Occupational Health (NIOH), Immunology and Microbiology Unit and the University of Johannesburg, Water and Health Research Centre (Doornfontein Campus). Reagents were stored and used in compliance with the recommendation as prescribed by the suppliers and used prior to their expiry dates. The selection of these laboratories was motivated by the fact that the NIOH conform to South African National Accreditation System (SANAS) standards.

2.5 Media preparation

All media used were sterilized by autoclaving at 120˚C for 15 minutes unless otherwise indicated.

2.5.1 Reagents and Buffer solutions

2.5.1.1 Page's amoebal saline (PAS)

Page's amoebal saline (PAS) was prepared by dissolving 120 mg NaCl, 4 mg $MgSO_4 \cdot 7H_2O$, 4 mg $CaCl_2.2H_2O$, 142 mg Na_2HPO_4 and 136 mg KH_2PO_4 in 1 L of distilled water. The solution was then stored at 4ºC and brought to room temperature before used.

2.5.2 PCR Buffers and solutions:

2.5.2.1 Celite:

Suspend 10 g celite in 50 ml H_20 and add 500 µl HCl (32% w/v). Then the celite solution in a beaker covered with foil, and the bottle is sterilised by autoclaving.

2.5.2.2 Lysis buffer:

Suspend 120 g GuSCN (Guanidinium thiocyanate) in a 100 ml of 0.1 M Tris-HCl at a pH of 6.4.

2.5.3 Culture Media

2.5.3.1 Nutrient agar

To prepare nutrient agar, 28 g of nutrient agar base (Oxoid, England) was dissolved in 1L of distilled water. The solution was sterilized by autoclaving for 15 minutes at 121˚C. Nutrient agar was allowed to cool to 55° after which it was poured into sterile petri dishes. Two nutrient agar plates were incubated at 37˚C for quality control purposes to check sterility.

2.5.3.2 Non-nutrient agar

Non-nutrient agar (NNA) plates were prepared by diluting 4 mg $MgSO_4.7H_2O$, 4 mg $CaCl_2.2H_2O$, 120 mg NaCl, 142 mg Na_2HPO_4, 136 mg KH_2PO_4 and 15 g of agar base (Merck, SA)) in 1L of Page's amoebal saline. The mixture was then boiled with frequent agitation to dissolve the agar completely before being poured into sterile petri dishes.

2.5.3.3 Xylose lysine desoxycholate (XLD) agar

Xylose lysine desoxycholate (XLD) agar base (Oxoid, England) was prepared by suspending 53 g of the dehydrated medium in 1L of distilled water, heated with frequent agitation until

the medium boiled and cooled in a water bath at 50ºC before being poured into plates. Two agar plates were incubated at 37°C for quality control purposes to check sterility.

2.5.3.4 Thiosulfate citrate bile sucrose (TCBS) agar

Thiosulfate citrate bile sucrose agar (TCBS) (Oxoid, England) was prepared by dissolving 88 g of the dehydrated medium in 1L of distilled water and sterilised by boiling. The medium was poured aseptically into Petri dishes. Two agar plates were incubated at 37ºC to check for sterility. In compliance to the manufacturer's instructions, this media was not autoclaved. The media was stored in the fridge at 2-10°C for no more than one month.

2.5.3.5 *Escherichia coli* chromogenic media

E. coli/coliform chromogenic agar plates, 28.1 g of dehydrated *Escherichia coli* chromogenic agar powder (Oxoid, England) was suspended in 1L of distilled water. The solution was boiled gently with agitation to dissolve completely before being cooled to 50ºC. The content was poured aseptically into petri dishes. Two agar plates were incubated at 37ºC to check for sterility. In compliance to the manufacturer's instructions, this media was not autoclaved. The media was stored in the fridge at 2-10°C for not more than a month if not used.

2.5.3.6 Buffered charcoal yeast extract (BCYE) agar.

For buffered charcoal yeast extract agar preparation, 12. 5 g BCYE agar base (Oxoid, England) was dissolved in 450 m1 distilled water by boiling gently. The medium was sterilized by autoclaving at 121ºC for 15 minutes. The medium was allowed to cool to 50ºC and 50 ml of BCYE supplement (Oxoid, England) was aseptically added and mixed thoroughly before being poured into sterile petri dishes. Two agar plates were incubated at 37ºC to check for sterility. We used a type strain of *L. pneumophila* (ATCC 33152) as a positive control and sterile distilled water was used as a negative control. The plates were refrigerated for no more than two weeks before use.

2.5.3.7 Middlebrook 7H10 medium

Middlebrook 7H10 OADC agar preparation was done by thoroughly dissolving 4.7 g of the Middlebrook 7H10 OADC agar base (Becton, USA) in 900 ml purified water containing 2 ml glycerol, heated with frequent agitation before being boiled for 1 minute to completely dissolve the powder. The medium was then autoclaved at 121ºC for 10 minutes and 100 ml of

the Middlebrook OADC *Enrichment* was aseptically added when the medium was cooled to between 45-55ºC.

2.6 Preparation of co-culture media

2.6.1 Non-nutrient-*Escherichia coli* plates

One colony of a type strain of *Escherichia coli* (ATCC 25922) was inoculated onto a nutrient agar plate, spread for single colonies and incubated overnight at 37ºC. The plate was then stored in a refrigerator and sub-cultured every two weeks. *Escherichia coli* bacteria were recovered from the plate with a sterile swab and suspended in sterile distilled water. The suspension was heat treated in a water bath for 15 minutes at 100°C to inactivate the bacteria. Three drops of heat killed *Escherichia coli* suspension were then inoculated onto the non-nutrient agar and spread evenly with a sterile swab. Two nutrient agar plates were also inoculated with this suspension and incubated at 37°C overnight to confirm that no organisms were viable in the suspension used to prepare the NNA-*Escherichia coli* plates.

2.7 Sample Collection and Transport

Samples were collected in new screw-capped polypropylene bottles early in the morning. The water temperature and pH were measured during sample collection. The samples were transported to the NIOH laboratory and processed on the day of collection.

2.8 Sample Concentration

On arrival at the NIOH laboratory the water samples (500 ml) were filtered through 0.45µm nitro- cellulose membranes to concentrate the sample.

2.9 Amoebal Enrichment

2.9.1 Non-nutrient agar *Escherichia coli* subculture

The filter membranes were then placed upside down onto non-nutrient agar-*Escherichia coli* plates prior to the addition of a drop of Page's amoebal saline (PAS) to aid the movement of the amoebae present in the samples. The plates were left at room temperature for 10 minutes to settle and subsequently incubated aerobically in plastic bags (to avoid desiccation) at 33°C. The plates were checked daily, using a light microscope with a 10x magnification, for the

morphological appearance of amoebal trophozoites and cysts. *Acanthamoeba* species were presumptively identified by both the polygonal shaped walls in the cyst form and the finger-like acanthapodia in the trophozoite form in all the samples. Plates without growth were re-incubated for at least 3 weeks before reporting a negative result.

To purify the amoebae observed on the agar plates, small agar plugs were aseptically cut from the areas on the plate where amoeba trophozoites and/or cysts were observed. The agar plugs were placed upside down onto fresh non-nutrient agar-*Escherichia coli* plates with a drop of Page's amoebal saline (PAS) subsequently added. The plates were left at room temperature for 10 minutes to settle, then again incubated at 33°C.

2.9.2 Microtitre plate isolation

After at least three sub-cultures, the amoebae cells were harvested by gently scraping the agar surface. The cells were then suspended in 2 ml PAS and washed three times by centrifugation at 1000 x g for 15 minutes where after 100 µl of the harvested pellets was inoculated into a 24 well, flat bottomed microtitre plate (Nunc, USA) containing 1ml PAS. The microtitre plates were incubated at 33°C and examined daily using an inverted microscope (Leica, Germany) with a 40x objective for the morphological appearance of amoebal trophozoites and cysts containing intracellular bacteria, or alternatively for wells containing disintegrated amoebal cells and large numbers of bacteria. Preliminary classification and enumeration of the presumptive type of FLA present were done based on cyst morphology.

Wells containing intracellular bacteria or disrupted amoebal cells were considered presumptively positive for amoeba resistant bacteria. To isolate potential intra-amoebal ARB, the amoebae cells were harvested by scraping the bottom of the microtitre well and re-suspending the contents in 2 ml PAS. The cells were centrifuged at 1000 x g for 15 minutes at room temperature. The supernatant was then removed leaving approximately 1 ml covering the pellets. The pellets were washed three times with PAS, then vortexed for 30 seconds to suspend the pellet after which 10µl portions were heat-fixed on three microscope slides and Gram staining, Ziehl Neelsen (ZN) acid-fast staining and Giemsa staining were performed for each sample. A further 100 µl of the suspension of each isolate was diluted with 600 µl PAS and frozen at -20˚C for molecular studies that were conducted according to Greub *et al.* (2006) as described in paragraph 2.12 below.

Depending on the results obtained from staining, the amoebae cells were inoculated onto selective media to detect *Legionella* species (BCYE), environmental *Mycobacteria*

(Middlebrook 7H10), *Salmonella species*, *Shigella species* and *enterobacteriaceae* (XLD), *Vibrio* species (TCBS) and *Esherichia coli* (chromogenic E. coli/ Coliform agar) potentially present in the samples. Figures 2.2 and 2.3 below show flow diagrams of the amoebal enrichment and confirmation tests conducted, respectively.

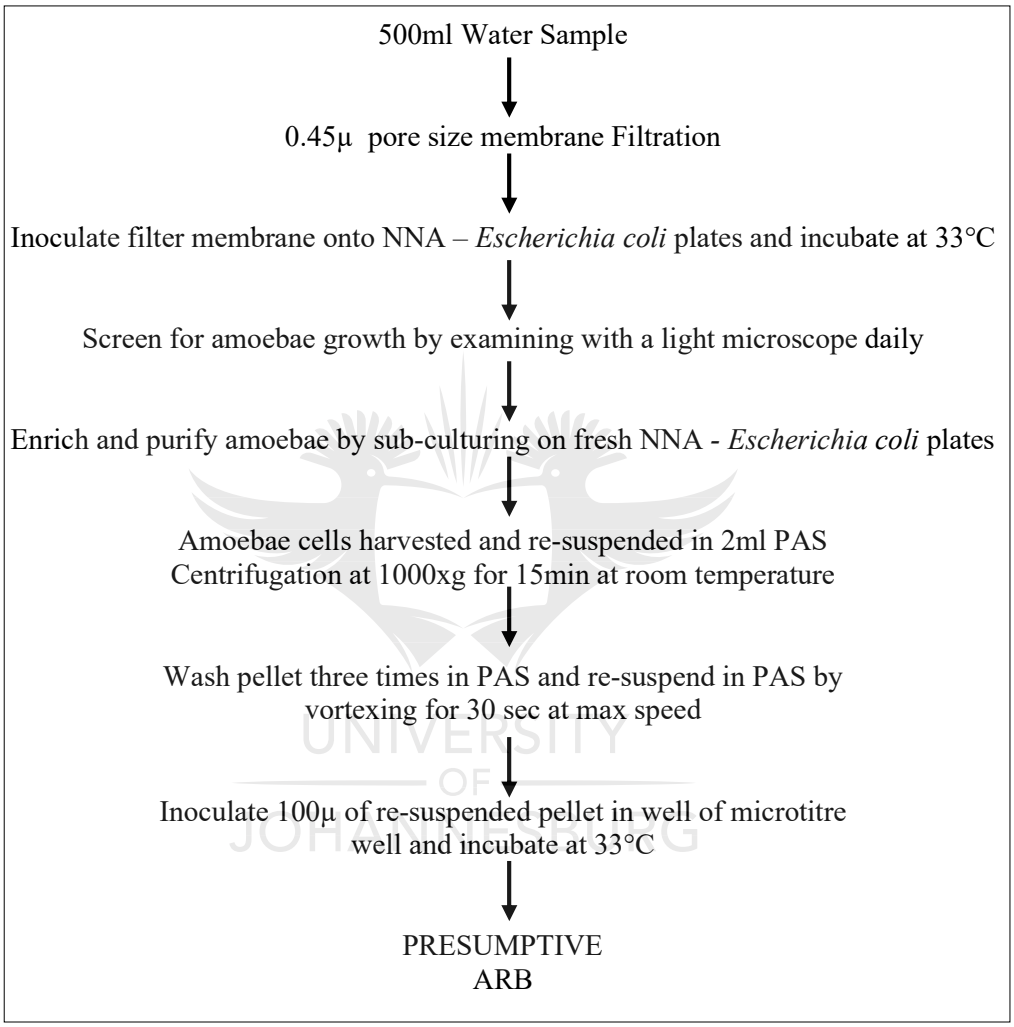


Figure 2.3: Flow diagram of amoebal enrichment technique used to isolate ARB from water samples

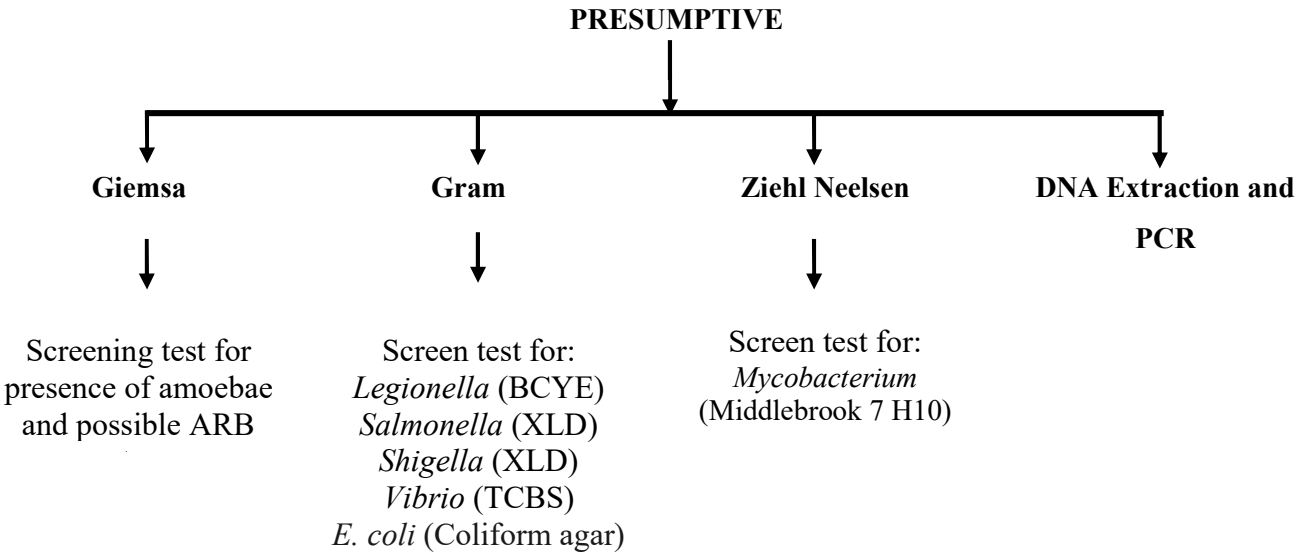


Figure 2.4: Flow diagram of ARB confirmation tests

2.10 MICROSCOPY

2.10.1 Preparation of slides

After releasing the ARB from amoebae, 50 µl of each sample suspension was transferred to a sterile glass slide using a micropipette. Three slides were prepared per sample, one each for Gram, Giemsa and acid-fast staining. The samples were heat fixed on slides by slowly passing it three times through the flame of a Bunsen burner, covered and stored at room temperature before staining to detect intracellular, Gram positive/negative and acid-fast bacilli using Giemsa, Gram and ZN stains respectively. Stained slides were air dried and examined under oil immersion lens at 100 x magnification on a light microscope (Olympus 9L08072, Japan).

2.10.2 Staining Procedures

2.10.2.1 Gram staining

The smears were placed on a staining rack and covered with crystal violet solution (NHLS). It was allowed to act for five minutes then rinsed with distilled water. The slides were then covered with iodine solution for 1 minute, after which it was rinsed with distilled water and again covered with acetone-alcohol decolourizer (NHLS) for 10 seconds and rinsed until no violet colour was visible with distilled water. The slides were the covered in safranin counter stain (NHLS) for one minute after which they were rinsed for the last time in distilled water.

The smears were then blotted and allowed to air dry. The dried slides were examined under oil immersed 100x magnification light microscope (Olympus, USA). Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which are stained purple by crystal violet, whereas Gram-negative bacteria have a thinner layer (10% of cell wall), which are stained pink by the counter-stain.

2.10.2.2 Giemsa staining

The slides were placed on the staining rack and covered with methanol (NHLS) for 10 minutes to fix the smear and placed into freshly prepared May Grunwald's stain (NHLS, SA) (Diluted stain in 1:10 Sorensen's phosphate buffer (PH=6.8, NHLS, SA) for 15 minutes. Then slides were placed into freshly prepared Giemsa's stain for 15 minutes where after the slides were rinsed in a Sorensen's phosphate buffer and allowed to air dry (do not blot dry). Amoebae will be stained blue, showing bacteria in the vacuoles.

2.10.2.3 Ziehl Neelsen staining

The slides were placed on staining rack and then flooded with carbol fuschin (NHLS SA) which was left on for 5 minutes. The slides were flamed and carbol fuschin were replenished if necessary. Afterwards slides were gently rinsed with distilled water and then decolourized with an acid alcohol solution (NHLS) for 30 minutes. This was rinsed off again with distilled water. The slides were then flooded with methylene blue (NHLS) and counter stain for 1-2 minutes after which excess stain was rinsed off with distilled water. The slides were blotted and allowed to air dry. The dried slides were examined under light microscope (Olympus, USA) equipped with a 100x oil immersion lens. The acid-fast bacilli have a lipid-rich cell wall that absorbs and retains phenol-dye solutions (eg. carbol fuchsin), resulting in reddish colour after Ziehl Neelsen staining.

2.11 CONFIRMATION

2.11.1 CULTURE

To culture isolates, 100 µl of presumptive ARB from the microtitre wells were inoculated onto plates containing standard *Escherichia coli*/c*oliform* Chromogenic media for *Escherichia coli*, standard Xylose Lysine Deoxicolate agar (XLD) for *Salmonella* and *Shigella* spp.,

standard Thiosulfate citrate bile sucrose (TCBS) agar for *Vibrio cholera*, standard Buffered charcoal yeast extract (BCYE) agar for *Legionella* spp. and standard Middlebrook 7H10 agar for *M. avium complex*. The plates were incubated at 37°C for 24-48 hours except in the case of *Legionella* spp. and *M. species* which were incubated for up to 3 weeks before a negative result were reported. (Table 2.2).

Table 2.2 ARB cultivation at 37°C on different selective media

ARB	Culture Media	Incubation temperature	Incubation period
Escherichia coli	*Escherichia coli* specific media	37°C	24 hours
Salmonella	XLD	37°C	24-48 hours
Shigella	XLD	37°C	24-48 hours
Vibrio cholera	TCBS	37°C	24-48 hours
Legionella species	BCYE	37°C	Up to 3 weeks
M. avium complex	Middlebrook 7H9	37°C	Up to 3 weeks

2.12 Molecular analysis of samples

DNA extraction and sequencing were carried out on 50 samples that tested positive for *Mycobacterium*. Polymerase Chain Reaction (PCR) analyses were used to fully identify the microorganisms present. In total, 50 samples were selected for PCR analysis to detect the presence of *V cholera*, Acanthamoeba species, *L. pneumophilla* and *M. avium complex*. Analysis was performed a week later by Inqaba Biotech Laboratories according to their in-house standard method.

2.12.1 DNA extraction

For DNA extraction, 700µl of samples suspension were centrifuged at 13000 rpm for 2 minutes. The supernatant was removed and 600µl PAS was added to the remaining 100µl covering the pellets. The content was vortexed before storage at -20°C.

2.12.2 DNA preparation

2.12.2.1 Column preparation

Column preparation was done according to the protocol originally described by Borodina *et al* (2003). To prepare the column the cap of 0.5ml PCR tube were cut off to leave a small bit behind and a red-hot needle were used to puncture several holes on bottom of tube. Further

silica membranes from GF/F filter paper were cut using 5 mm punch and 2 membranes were tightly inserted into the tube.

2.12.2.2 Spin column procedure:

The spin columns were placed in clean 2 ml tube and loaded with a third of the solution, after which it was then centrifuge for 30 seconds at 13000 RPM, after which the elute were discarded. This was repeated until the columns were fully loaded.

When columns were loaded 400 µl of wash buffer were added and the columns were centrifuged for 30 seconds at 13000 RPM and the elute were discarded. This process was repeated after which 400 µl 70 % EtOH were added to the columns, which was then again centrifuged for 30 seconds at 13000 RPM and the elute were again discarded. The alcohol step was also repeated once. The columns were then dried using centrifugation at 13000 RPM for 2 minutes after which the columns were transferred to clean tubes and a 100 µl AE buffer were added to the columns before it was incubated for 2 minutes at 65°C. After incubation columns were again centrifuged for 2 minutes, after which the DNA was ready to be used for PCR applications.

Table 2.3: Primer and Sequence for PCR

Primer name	Sequence	Reference
OmpW (*Vibrio*)	5'-CCACCTACCTTTAGCTTCACC-3'	Bisweswar et al.,2000
Ami6F1 (*Amoebae*)	5'-CCAGCTCCAATAGCGTATATT-3'	Thomas *et al*., 2006
Leg 225 (*Legionella*)	5'-AAGATTAGCCTGCGTCCGAT-3'	Thomas *et al*., 2006
Myco66f (*M avium complex*)	5'-CATGCAAGTCGAAXGGAAA-3'	Thomas *et al*., 2006
Chlamydia	5'-CGGCGTGGATGAGGCAT-3'	Everett *et al*., 1999

The samples which were positive for the presence of *M avium complex* species during sub-culturing, staining, selective media culturing was selected for PCR analyses.

2.12.3 Data analysis.

All results were captured in a Microsoft Excel spreadsheet and later imported into Microsoft Access for crosstab analysis and specific data mining. Crosstab analysis is a special type of

sort procedure used in databases to reorganise and re-categorise data and analyse large amounts of data in different ways.

Statistical analysis was performed by using Sigma Stat 4.0, University of Johannesburg. Where applicable, data was analysed using a one-way and two-way analysis of variance (ANOVA), 95% confidence level ($p<0.05$) where accepted as statistical significant change and p values were reported to a maximum of three decimal points.

Simple descriptive statistics (mean, standard deviation) were done using Excel from Microsoft Office 2016® and SigmaPlot 14.0. The results were generally expressed as the mean ± the range. The presence of ARB and FLA was reported in frequency tables.

CHAPTER 3

3 RESULTS

3.1 Introduction

Samples were collected from a wastewater treatment plant during the four seasons and all stages of the treatment process. These samples were tested for physico-chemical paramaters (temperatures and pH) as well as screened for free living amoebae and amoeba resistant bacteria. A total number of 172 samples were collected: 41 in autumn, 43 in winter, 44 in spring and 44 in summer.

3.2 Physico-chemical parameters

3.2.1 pH

There was no significant difference in the pH of the samples tested among the seasons covered in this study. The pH ranged from 6.33 to 8.13 and was not seasonally dependent. However, the pH differed considerably amongst the different processes (Table 3.1). Although there were considerable differences in the pH among some of the processes, these differences were not seasonally nor temperature dependant and therefore should be considered process specific. The average pH of the primary (pH 7.15 ± 0.27) and secondary treatment (pH 7.14 ± 0.17) processes were not significantly different but the tertiary treatment process (pH 7.31 ± 0.29) had a significantly higher pH than the primary ($p < 0.01$) and secondary treatments ($p<0.001$). The average pH at the beginning of the treatment process was 7.24 ranging from 6.77 to 7.70 (SD = 0.22) in Process1 (during primary treatment) and then decreased to 7 (6.81-7.18, SD = 0.11) in Process 4 (during secondary treatment) after which it increased again and peaked in Processes 6 and 7 (during tertiary treatment), with pH values of 7.36 (6.95 -7.89) and 7.35 (6.91-7.76) respectively. The pH of the final process was (7.29; 6.33-8.13) which was very similar to the pH observed at the first process (Table 3.1). These differences in pH did not influence the presence of ARB or FLA.

3.2.2 Temperature

Water temperatures ranged from as low as 6.6°C in winter to as high as 27.7°C during the summer with a standard deviation (SD) of 5.5°C, which was seasonally dependent. (Table 3.1)

Table 3.1 Physico-chemical parameters in the water treatment processes.

			WATER TEMPERATURE (°C)		WATER pH	
TREATMENT	**PROCESS**	**n**	**RANGE (MIN-MAX)**	**MEAN (SD)**	**RANGE (MIN-MAX)**	**MEAN (SD)**
PRIMARY TREATMENT	**PROCESS 1: Untreated sewage**	16	12.1-25.8	20.31 (4.21)	6.77-7.70	7.24 (0.22)
	PROCESS 2: Sewage entering Bioreactor.	20	11.5-25.7	19.70 (4.70)	6.65-7.90	7.08 (0.29)
TOTAL		**36**	**11.5-25.8**	**19.97 (4.43)**	**6.65-7.90**	**7.15 (0.27)**
SECONDARY TREATMENT	**PROCESS 3: No Oxygen Available**	16	9.4-25.9	19.07 (5.04)	6.90-7.30	7.08 (0.12)
	PROCESS 4: Bound oxygen available in a form of Nitrate/ Denitrification Zone	16	9.1-27.6	19.53 (5.57)	6.81-7.18	7.00 (0.11)
	PROCESS 5: Oxygenated zone/Nitrification Zone	32	8.9-27.6	18.87 (5.20)	6.91-7.80	7.23 (0.17)
TOTAL		**64**	**8.9-27.6**	**19.08 (5.18)**	**6.81-7.80**	**7.14 (0.17)**
TERTIARY TREATMENT	**PROCESS 6: Bioreactor effluent after nutrient removal**	16	13.4-26.2	19.72 (4.05)	6.95 -7.89	7.36 (0.22)
	PROCESS 7: Treated Sewage	11	10.9-27.7	19.59 (4.75)	6.91-7.76	7.35 (0.24)
	PROCESS 8: Maturation Pond	45	6.6-27.7	18.47 (6.13)	6.33-8.13	7.29 (0.33)
TOTAL		72	6.6-27.7	18.92 (5.50)	6.33-8.13	7.31 (0.29

Table 3.2: Water temperature and pH during sampling for different seasons.

		WATER TEMPERATURE (°C)		WATER pH	
SAMPLING SEASON	**n**	**RANGE (MIN-MAX)**	**MEAN (SD)**	**RANGE (MIN-MAX)**	**MEAN (SD)**
Autumn	41	12.6-22.9	18.31 (2.78)	6.33-7.9	7.19 (0.33)
Winter	43	6.6-18.2	12.45 (2.64)	6.65-8.13	7.28 (0.29)
Spring	44	12.7 -23.6	20.56 (1.99)	6.67-7.76	7.20 (0.20)
Summer	44	21.8 -27.7	25.26 (1.45)	6.77-7.70	7.18 (0.20)
Total	**172**	**6.6 -27.7**	**19.2 (5.16)**	**6.33-8.13**	**7.21(0.26)**

3.3 Microscopic screening for the presence of FLA

The samples were screened for the presence of free-living amoebae by light- and inverted microscopy. Cysts with the characteristic *Acanthamoeba* cyst shape were recorded as "*Acanthamoeba* cysts"; all other cysts are referred to as "round cysts" as they could not be used for the presumptive identification of specific genera of FLA.

Of the 172 samples, all presented with either trophozoites or cysts or both life forms of FLA. Amoeba trophozoites were observed in 165 (96%) of the samples and in 22 of these samples typical *Acanthamoeba* cysts were observed (18 of these were accompanied by trophozoites, 3 only presented with cysts and 1 had trophozoites and round cysts present as well).

The 22 samples that presented with *Acanthamoeba* cysts had a pH range of 6.75 to 7.9 and a sample temperature ranging between 15.1 to 25.4°C. Free-living amoeba and/or their cysts were present throughout the treatment plant, but differences were observed among the treatment processes. Cysts representing those of *Acanthamoeba* species were observed in all the processes except Process 7 (Treated Sewage).

The processes with the highest number of positive samples were Reactor Feed (Sewage Entering the Bioreactor, Process 2: n=3), Anoxic Zone (No Oxygen Available, Process 3: n=3), Bound Oxygen Available (Nitrate/Denitrification Zone, Process 4: n=4), and the Bioreactor Effluent (after nutrient removal, Process 6: n=4). See table 3.3

The only seasonal difference was recorded during the autumn run when 51.2% of the samples (n=21) screened positive for *Acanthamoeba* cysts.

Table 3.3 The life cycle forms of amoeba observed in samples at different stages of the treatment process. (Percentages in process column represent the percentage of samples tested per process and not of total)

Stage of life	Primary treatment		Secondary treatment			Tertiary treatment			Total Positive Samples (n=172)
	Process 1 (n=16)	Process 2 (n=20)	Process 3 (n=16)	Process 4 (n=16)	Process 5 (n=32)	Process 6 (n=16)	Process 7 (n=11)	Process 8 (n=45)	
Trophozoites with both Acanthamoeba cysts and Round Cysts	0 (0,0)	0 (0,0)	0(0,0)	0 (0,0)	0 (0,0)	1(6.3)	0 (0,0)	0 (0.0)	1(0.6)
Trophozoites and Acanthamoeba cysts	1 (6.3)	3 (15.0)	3 (18.8)	4 (25.0)	1 (3.1)	4 (25.0)	0 (0.0)	2 (4.4)	18 (10.5)
Trophozoites and Round Cysts	14 (87.5)	16 (80.0)	13 (81.2)	12 (75.0)	28 (87.5)	11 (68.8)	10 (90.9)	42 (93.4)	146 (84.9)
Acanthamoeba cysts only	1 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.1)	0 (0)	0 (0)	1 (2.2)	3 (1.7)
Round cysts only	0 (0.0)	1 (5.0)	0 (0.0)	0 (0.0)	2 (6.3%)	0 (0.0)	1 (9.1)	0 (0-0)	4 (2.3)

3.4 Microscopic screening for the presence of ARB

Slides from all 172 samples were prepared for Giemsa, Gram and Ziehl Neelsen (ZN) staining to observe amoebae and intercellular bacteria, Gram negative bacteria and *Mycobacterium* species respectively. The Giemsa stain showed that 95.9% of the samples stained to be positive for amoebae trophozoites and/or cysts (Table 3.4).

Table 3.4: Differential stains for free living amoeba.

	Gram stain	Giemsa	ZN Stain
Positive	0(0%)	165(95.9%)	52(30.2%)
Negative	172(100%)	7(4.1%)	120(69.8%)

During the microscopic examination of all the 172 samples, 29 (17%) had only intracellular bacteria present. All other samples showed only extracellular bacteria and therefore were considered negative for ARB. One sample had both intra- and extracellular bacteria.

Of the 29 samples that tested positive for only intracellular bacteria, 23 (79%) were collected during Autumn and the other 6 samples were collected in the other seasons (2 per season). The sample that tested positive for both intra and extracellular bacteria was also collected during Autumn.

The 29 samples that tested positive for only ARB 26 (90%) had trophozoites present as well as cysts, and 20 (69%) presented with acanthamoeba cysts and 9 (31%) presented with round cysts. The sample that tested positive for both ARB and extracellular bacteria presented with only round cysts.

The ARB positive samples had a pH range from 6.75 to 7.9. However, the pH range for the majority of samples that tested positive for acanthamoeba cyst and ARB was 6.75 to 7.47 (80%, n=18) and those that had round cysts and ARB 7.03 -7.45 (n=8)

The temperature range of samples that tested positive for ARB was 11.7 to 25.9° C. Furthermore, all samples that were positive for acanthamoeba and ARB were spread over a narrower temperature range of 15.1 to 22.1°C (100%; n=20) were as those that were positive for round cysts and ARB were found over the full temperature range of 11.7 to 25.9°C, with only 44% falling into the temperature range for the acanthamoeba positive samples.

All processes tested positive for ARB harboured in either acanthamoeba or round cysts.
The Gram stain showed that all the bacteria found inside the amoeba to be Gram negative, and only 30.2% of these bacteria to be acid fast as well, indicating possible *Mycobacterium* species (table 3.5).

Table 3.5: Comparison of ZN Stain to Culture

	Total	Culture Positive	Culture Negative
ZN Positive	52(30.2%)	51(98.1%)	1(1.9%)
ZN Negative	120(69.8%)	46(38.3%)	74(61.7%)

3.5 Culture identification of ARB

After initial microscopic examination to identify bacteria as intra- or extra-cellular the bacteria were further identified using culture methods as described in Chapter 2. The ARB organisms present in the different processes are summarized in Table 3.6.

Table 3.6 Total bacteria cultured per treatment process

Bacteria cultured	Primary treatment		Secondary treatment			Tertiary treatment			Total Positive Samples (n=172)
	Process 1 (n=16)	Process 2 (n=20)	Process 3 (n=16)	Process 4 (n=16)	Process 5 (n=32)	Process 6 (n=16)	Process 7 (n=11)	Process 8 (n=45)	
Escherichia coli	16 (100)	20 (100)	16 (100)	16 (100)	32 (100)	16 (100)	11 (100)	45 (100)	172(100)
Salmonella species	0	2 (10)	2 (12.5)	0	1 (3.1)	2 (12.6)	0	6 (13.3)	13(7.6)
Shigella species	16 (100)	20 (100)	16 (100)	16 (100)	32 (100)	15 (93.8)	11 (100)	45 (100)	171(99,4)
Vibrio species	0	0	0	0	0	1 (6.3)	0	0	1(0.6)
Legionella species	0	1 (5.0)	1 (6.3)	0	1 (3.1)	2 (6.3)	0	3 (6.7)	8 (4.7)
Mycobacterium species	10 (62.5)	12 (60.0)	7 (43.8)	10 (62.5)	16 (50.0)	8(50)	4 (36.4)	30 (66.7)	97(56.4)

Percentages are shown in parentheses.

The 172 samples all cultured positive for *Escherichia coli* (100%) and 171 (99.4%) cultured positive for *Shigella* species. *Mycobacterium* species were cultured in 97 (56.4%) of the samples. Although they were distributed throughout all processes, they were most prevalent in the samples collected from Process 2 (reactor feed), Process 4 (bound oxygen present) and Process 8 (maturation ponds). The other bacteria tested for were present in less than 10% of the samples with *Salmonella* species in 13 (7.6%), *Vibrio* species in 1(0.6%) and *Legionella* species in 8 (4.7%). Samples from the final effluent tested positive for all organisms, whereas samples from the maturation pond, the reactor feed (Sewage entering the Bioreactor), anaerobic zone (No oxygen available), aeration zone (Oxygenated zone/Nitrification zone) tested positive for all but *V. cholera*. However, samples from the reactor effluent (treated sewage), the outfall sewer (untreated sewage), and the anoxic zone of the bioreactor (Bound oxygen available in a form of Nitrate/Denitrification zone) only tested positive for *Escherichia coli, Shigela* and *M. avium*. (See Table 3.6)

When we look at only the samples that were positive for ARB they all presented with both *E coli* and *Shigella* regardless if the samples had acanthamoeba or round cysts present and these were found across all processes. *Salmonella* was only cultured in 2 of the 30 ARB positive samples and one was associated with acanthamoeba cysts and one with round cysts and was found in the No Oxygen process and the other in the maturation pond, Only one of the ARB positive samples cultured positive for *Legionella* and this was associated with round cysts and found in a sample collected from the no oxygen process. Our cultures for *Mycobacterium avium* produce positive results in 14 of the ARB positive samples of which 8 was associated with acanthamoeba cysts and 6 with round cysts, these were collected from all processes except the treated sewage process.

3.5.1 Escherichia coli

Overall only 30 (17.4%) of all sample presented with intracellular bacteria with the steps where no oxygen (Process 3) were available showing 7(43.8%) of the samples with intracellular bacteria, were as the oxygenated zone presented with the lowest amount of samples with intracellular bacteria (n=2;6.3%).

Table 3.7: Samples with intracellular bacteria

	Primary treatment		Secondary treatment			Tertiary treatment			Total Positive Samples (n=172)
	Process 1 (n=16)	Process 2 (n=20)	Process 3 (n=16)	Process 4 (n=16)	Process 5 (n=32)	Process 6 (n=16)	Process 7 (n=11)	Process 8 (n=45)	
Number samples with Intracellular Bacteria	4 (25.0)	4 (20.0)	7(43.8)	4 (25.0)	2 (6.3)	4(25.0)	1 (9.1)	4 (8.9)	30(17.4)

All samples that had intracellular bacteria cultured positive for *Escherichia coli*. See Table 3.7. The majority of intracellular bacteria were found in presumptive acanthamoeba cysts or trophozoites (n= 20; 66.7%). Samples presenting with both trophozoites and cysts accounted for 86.7% (n=26) of samples with intracellular bacteria and only 3 (10%) of the samples presenting with intracellular bacteria had only presumptive acanthamoeba cysts. Furthermore, 9 (33.3%) of samples, presenting with intracellular bacteria, were positive for round cysts and trophozoites of other acanthamoeba species. As was the case with the samples presenting with presumptive acanthamoeba the largest number of samples with round cyst with intracellular bacteria had presented with trophozoites (30%) as well. Presumptive acanthamoeba and their cysts were found exclusively in the processes where Oxygen were available in a form of nitrate (denitrification), oxygenated zone (Nitrification zone) and the bioreactor effluent after nutrient removal. Whereas round cysts and their trophozoites were exclusively present in only the treated sewage. In the untreated sewage both species of amoeba was present. Presumptive acanthamoeba were the dominant form found in the samples from the maturation pond and where the sewage entered the bioreactor. However, the round cyst and their trophozoites were found to be more prominent in the samples from the processes where no oxygen were present.

3.5.2 Shigella

Shigella was cultured in 99.4% of all samples tested and as in the case for *E coli* 17.4% presented with cysts and intracellular bacteria (shigella). Presumptive acanthamoeba accounted again for 11.6% of amoeba with intracellular bacteria whereas round cysts only

accounted for 5.8%. Furthermore, of the samples that showed extracellular bacteria only 0.6% had presumptive acanthamoeba cysts present whereas 81.4% had round cysts present.

Table 3.8: Intracellular bacteria testing positive for *Shigella*

	Primary treatment		Secondary treatment			Tertiary treatment			Total Positive Samples (n=172)
	Process 1 (n=16)	Process 2 (n=20)	Process 3 (n=16)	Process 4 (n=16)	Process 5 (n=32)	Process 6 (n=16)	Process 7 (n=11)	Process 8 (n=45)	
Number samples with Intracellular Bacteria	4 (25.0)	4 (20.0)	7(43.8)	4 (25.0)	2 (6.3)	4(25.0)	1 (9.1)	4 (8.9)	30(17.4)

The *shigela* cultures revealed the same pattern as that of *Escherichia coli* see paragraph 3.4.1 and Table 3.7

3.5.3 Salmonella

Only 7.6% of samples culture positive for *salmonella* and out of these only 15.4% had cysts with intracellular bacteria present, 7.7% with presumptive acanthamoeba and 7.7% with round cysts. The rest of the positive samples can be accounted for by samples that presented with round cysts with extracellular bacteria (76.9%) with only 7.7% having presumptive acanthamoeba with extracellular bacteria.

Table 3.9: Intracellular bacteria testing positive for *Salmonella*

	Primary treatment		Secondary treatment			Tertiary treatment			Total Positive Samples (n=172)
	Process 1 (n=16)	Process 2 (n=20)	Process 3 (n=16)	Process 4 (n=16)	Process 5 (n=32)	Process 6 (n=16)	Process 7 (n=11)	Process 8 (n=45)	
Number samples with Intracellular Bacteria	0 (0.0)	0 (0.0)	1(6.3)	0 (0.0)	0 (0.0)	0(0.0)	0(0.0)	1 (2.2)	2(1.2)

Although only two samples tested positive for intracellular *salmonella* the samples were from two different processes and presented with presumptive acanthamoeba cysts and trophozoites in the sample from the maturation pond and with round cyst and their trophozoites in the sample from the process were there was no oxygen was present. These are the processes were these amoeba forms were the most abundant. (As summarised in table 3.9)

3.5.4 Mycobacterium avium

The ZN stain was very specific with 51(98,1%) of the 52 specimens that test positive with ZN staining yielding positive cultures as well. However, from the 120 the samples that tested negative with ZN staining 46(38,3%) cultured positive. This could have been due to low initial numbers of bacteria present in the original sample that were more easily picked up during culture as shown in Table 3.5.

Furthermore, from the 56.4% samples that culture positive for *Mycobacterium Avium* 1% came from samples that had only presumptive acanthamoeba cyst with intracellular bacteria present and 7.3% from samples that had both trophozoites and presumptive acanthamoeba cysts with intracellular bacteria. A further 5.2% had round cysts and trophozoites with intracellular bacteria and 1 % had only round cysts with both intra and extra cellular bacteria present. Intracellular bacteria in cysts accounted for 13.5% of the *Mycobaterium* species positive cultured samples. The other 85.5% of sample had round cysts and trophozoites and extracellular bacteria.

Table 3.10: Presence of *M. avium* in the different processes.

	Primary treatment		Secondary treatment			Tertiary treatment			Total Positive Samples (n=172)
	Process 1 (n=16)	Process 2 (n=20)	Process 3 (n=16)	Process 4 (n=16)	Process 5 (n=32)	Process 6 (n=16)	Process 7 (n=11)	Process 8 (n=45)	
Number samples with Intracellular Bacteria	1 (6.3)	1 (5.0)	3(18.8)	3 (18.8)	1 (3.1)	1(6.3)	0(0.0)	4 (8.9)	14(8.1)

Mycobacterium avium tested positive in 8.1 % of samples with intracellular bacteria. Furthermore 12 (85.7%) samples with intracellular bacteria testing positive for *mycobacterium* were found in samples where both trophozoites' and cysts were present, with presumptive acanthamoeba being the dominant species (8/14; 57.1%). *Mycobacterium* were found in all processes except in the treated sewage (3.1-18.8%). The highest incidence of samples testing positive for *Mycobacterium* were the process with no oxygen (Process 3;18.8%), the process with oxygen present in the form of nitrate (Process 4;18.8%).(Refer to Table 3.10)

3.5.5 Legionella

Only 4.7% (8 samples) of samples cultured positive for *Legionella* of which only 14.3% (1 sample) had round cysts with intracellular bacteria present. All 7 samples had round cysts

present but 6 of the 7 had only extracellular bacteria present. It was only samples collected in the winter and summer that tested positive for *legionella.* See Table 3.11

Table 3.11: Presence of *Legionella* in the different processes.

	Primary treatment		Secondary treatment			Tertiary treatment			Total Positive Samples (n=172)
	Process 1 (n=16)	Process 2 (n=20)	Process 3 (n=16)	Process 4 (n=16)	Process 5 (n=32)	Process 6 (n=16)	Process 7 (n=11)	Process 8 (n=45)	
Number samples with Intracellular Bacteria	0 (0.0)	0 (0.0)	1(6.3)	0 (0.0)	0(0.0)	0(0.0)	0(0.0)	0 (0.0)	1(0.6)

3.5.6 Vibrio cholera

Where *Vibrio cholera* was concerned only one sample (0.6%) tested positive, this sample was collected from process 6 (Bioreactor effluent after nutrient removal). However, these bacteria were found outside the amoeba host. See table 3.6

3.6 Polymerase Chain Reaction (PCR)

Of the 97 samples that cultured positive for *Mycobacterium* species 50 were randomly selected for PCR. The results are summarized in Table 3.12. Samples from all processes were represented in the 50 (29.1%) samples, 18.1-62.2% of samples of each process being selected for PCR. The pH range of the samples where between 6.33 to 8.13 and the sample temperature between 7.7 and 27.7.(See PCR Gels Appendix B). According to the results that we observed, some microscopically positive samples were negative after PCR, suggesting that all samples were supposed to be processed by PCR. However only 50 were processed by PCR, out of 172 samples due to financial constraints. Furthermore, some contamination was problematic during screening on selective media.

Table 3.12: PCR results reported per Process.

	Primary treatment		Secondary treatment			Tertiary treatment			Total positive Samples (n=172)
	Process 1 (n=16)	Process 2 (n=20)	Process 3 (n=16)	Process 4 (n=16)	Process 5 (n=32)	Process 6 (n=16)	Process 7 (n=11)	Process 8 (n=45)	
Number of sample for PCR	6 (37.5)	5 (25.0)	4(25.0)	4 (25.0)	8 (25.0)	4 (25.0)	2 (18.1)	28 (62.2)	50(29.1)
Acanthamoeba	0/6(0.0)	0/5 (0.0)	**1/4 (25.0)**	0/4(0.0)	**1/8 (12.5)**	0/4 (0.0)	0/2(0.0)	0/28 (0.0)	**2/50(4.0)**
Legionella species	0/6 (0.0)	**1/5 (20.0)**	**2/4(50)**	0/4 (0.0)	**1/8 (12.5)**	**1/4 (25.0)**	0/2 (0.0)	**8/28 (28.6)**	**9/50(18.0)**
Vibrio species	0/6(0.0)	0/5(0.0)	0/4(0.0)	0/4(0.0)	0/8(0.0)	0/4 (0.0)	0/2(0.0)	0/28(0.0)	0/50(0.0)
Mycobacterium species	0/6 (0.0)	0/5 (0.0)	0/4 (0.0)	0/4 (0.0)	0/8 (0.0)	**1/4(25)**	0/2 (0.0)	0/28 (0.0)	**1/50(2.0)**
Chlamydia	**1/6(16.7)**	**2/5(40.0)**	**4/4(100)**	**2/4(50.0)**	**2/8(25.0)**	**3/4 (75.0)**	**1/2(50.0)**	**6/28(21.4)**	**25/50(50.0)**

Only two of the 50 samples tested via PCR, tested positive for the specific acanthamoeba DNA sequence used. However, the samples tested showed that 9 of the 50 (18.0%) had *legionella* DNA present which correlated with that results found in the culture. Unfortunately, this was not true of the *M avium* PCR were only 1 of the 50 samples tested positive (2.0%) for M.avium.

3.6.1 Acanthamoebae

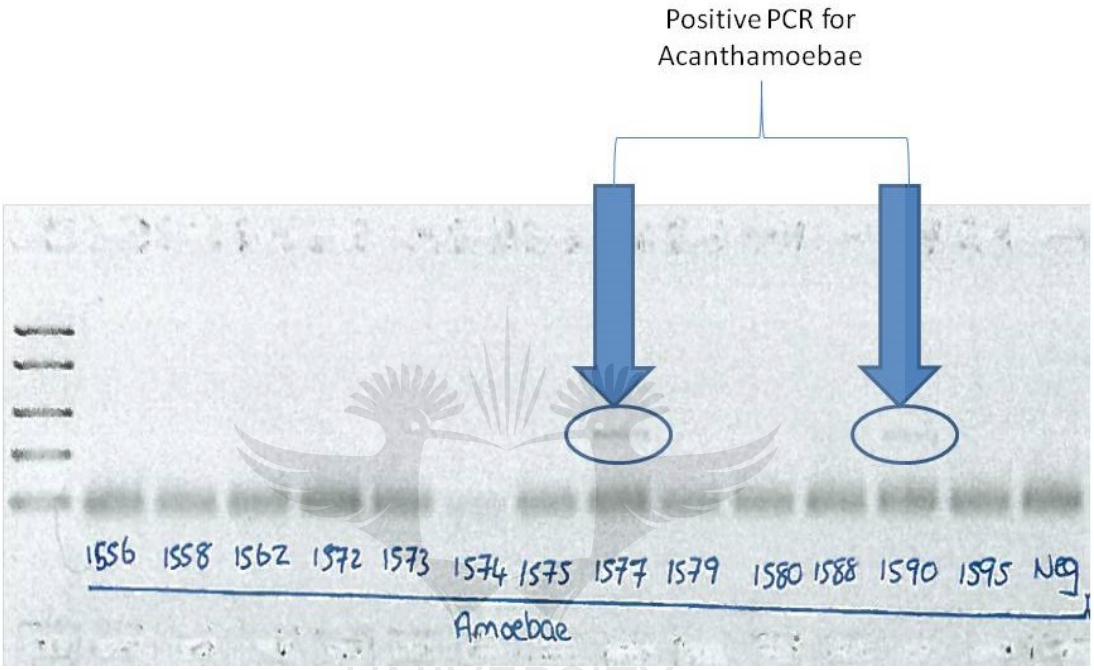


Figure 3.1 Positive PCR for Acantamoebae**.**

The two samples that tested positive for acanthamoeba DNA displayed trophozoites and round cysts under microscopical examination. One of these samples had intra cellular bacteria present and the other not. Further studies need to be done to get clarity on this phenomenon.

3.6.2 *Legionella* PCR

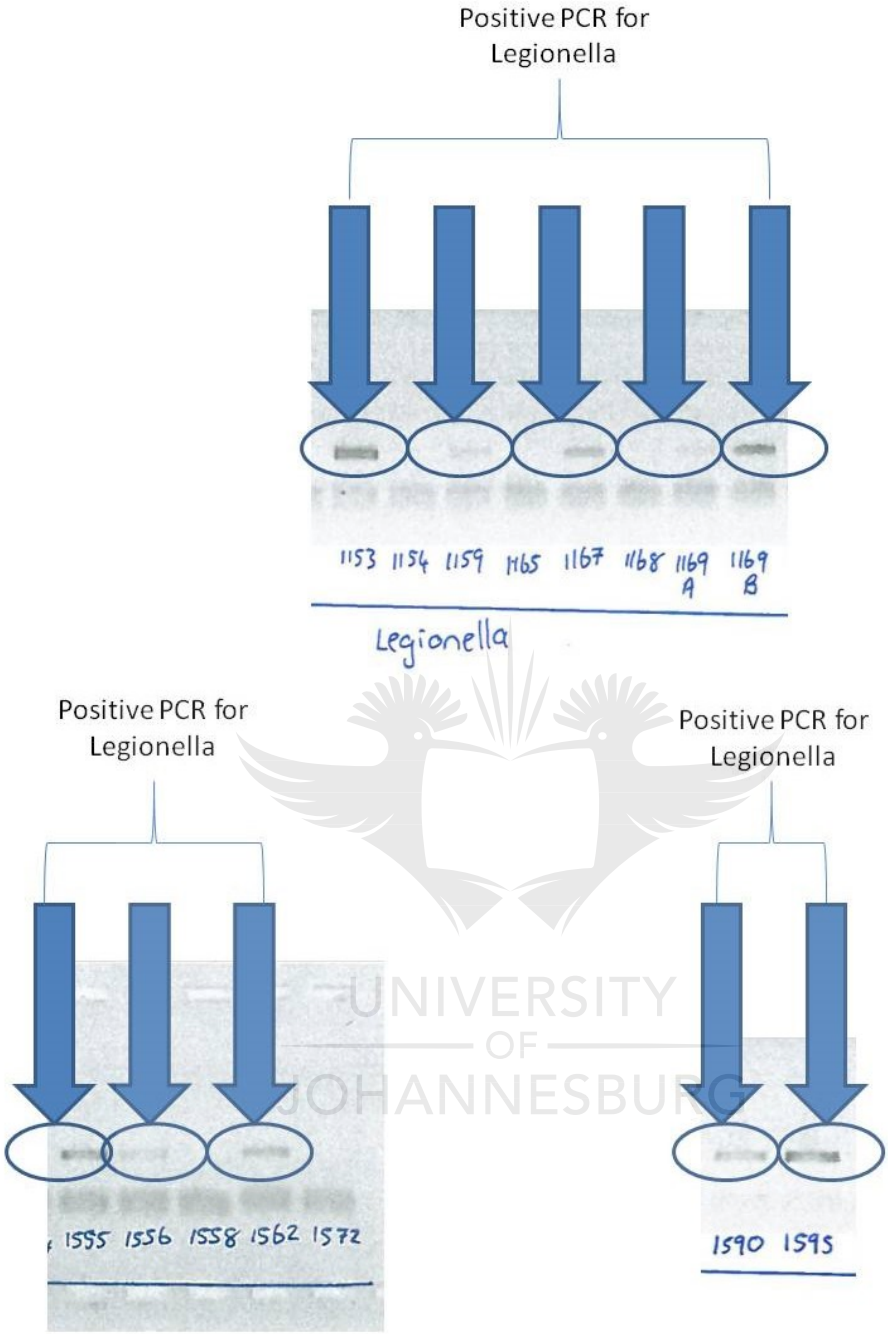


Figure 3.2: Positive PCR for *Legionella*.

The PCR for *legionella* identified two additional samples to have *legionella* present. However they were found external to the trophozoites and the round cyst present in these samples. All the samples that cultured positive for *legionella* were confirmed by PCR as being *legionella*. All these samples were collected during the winter run with water temperatures below 10°C.

3.6.3 *Mycobacterium* PCR

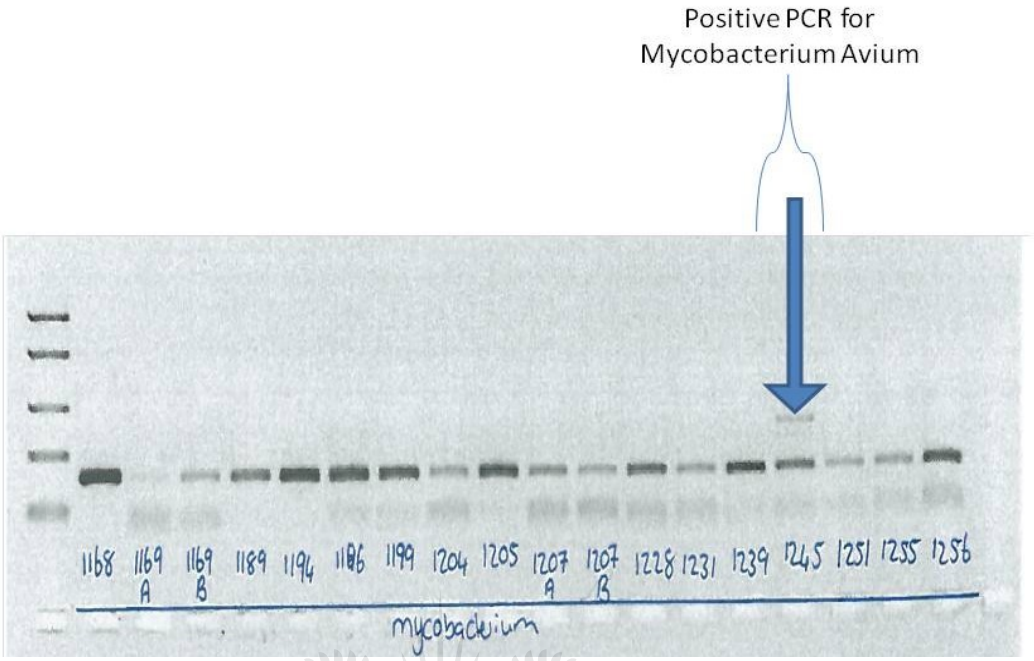
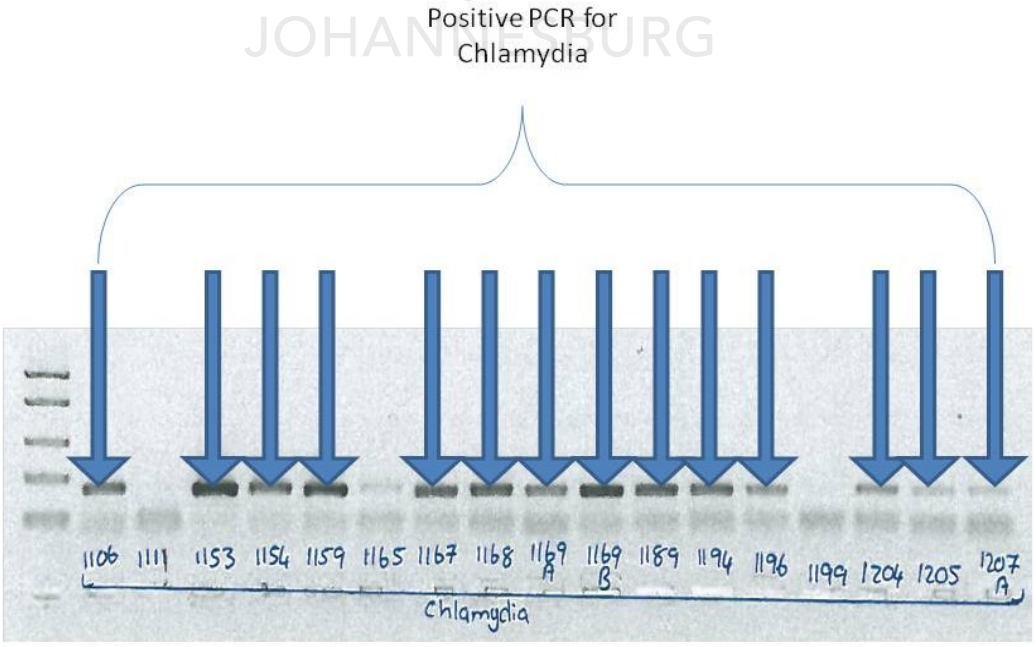


Figure 3.3: Positive PCR for Mycobacterium.

We found only one of the samples that culture positive to be confirmed via PCR to be *mycobacterium avium*. As to the identity of the other cultured *mycobacterium* further studies needs to be conducted to identify them.

3.6.4 *Chlamydia* PCR.



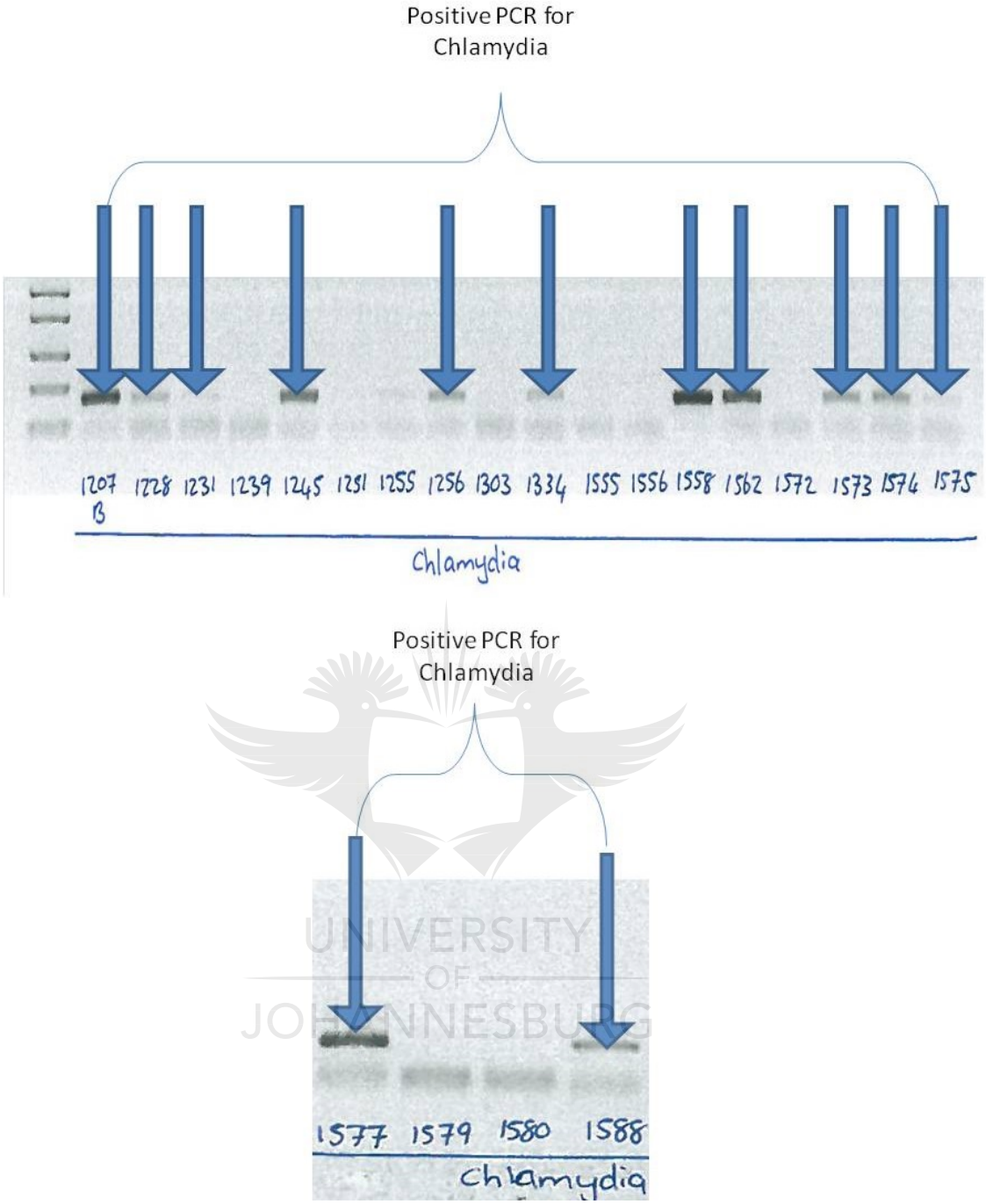


Figure 3.4: Positive PCR for *Chlamydia*.

Although *chlamydia* was not originally earmarked as part of this study, we found that it was quite common in the samples that were analysed via PCR with 50.0% of all sample testing positive via PCR. This fact will be the subject of further studies into ARB's in sewage. However, with PCR we cannot distinguish if these organisms were viable or not.

The 2 samples that presented with the intracellular bacteria was taken from the anaerobic zone (process 3) and Bioreactor effluent (process 6) of the plant and presented with trophozoites and round cysts and acanthamoeba cysts respectively.

CHAPTER 4

4 DISCUSSION AND CONCLUSION

4.1 Discussion

4.1.1 Introduction

In this study, we investigated the occurrence of amoeba resistant bacteria (ARB) in samples from a wastewater treatment plant in Johannesburg, South Africa. A broad range of staining methods, culture approaches and PCR tools were used for this study.

4.1.2 Environmental and physio-chemical parameters

Due to the large changes in the environmental conditions in the Highveld area temperature was considered as one of the variables in this study. There was a significant change in the water temperature during the period of investigation with temperatures ranging from 6.6°C to 27.7°C (see table 3.1). Although all the positive *Acanthamoeba* samples were found in a defined pH and temperature range neither pH nor temperature can be reported as a definite limiting or predictive factor in the detection of *Acanthamoeba*, as the samples that tested negative for *Acanthamoeba* and presented with other forms of FLA also had temperature and pH ranges that overlapped with the ranges found for the *Acanthamoeba* positive samples. Therefore, unlike other literature (Sakran *et.al*., 2019), the temperature did not affect any of the other results with the pH showing no seasonal variation.

4.1.3 Free living Amoeba

Of the 172 samples analysed FLA's were cultured from all samples with only 12.8% of samples showing the presence of presumptive acanthamoeba via cysts formation. This however could not be confirmed in the PCR analysis. The two samples that tested positive for *acanthamoeba* DNA were thought to have only round cysts and trophozoites of other amoeba species and only one of these samples presented with a range of ARB's (all except *V. cholera)* when cultured and *Legionella* were also confirmed via PCR. We therefore would like to suggest further investigation into the PCR techniques especially that of obtaining DNA for the confirmation of acanthamoeba. In other studies, it was shown that morphological classification could be flawed and therefore for the sake of this study we will refer to the samples that were identified by morphology as acanthamoeba as presumptive acanthamoeba. However, the positive identification of the amoeba present in the samples is of lesser importance than the fact of their presence and their ability to harbour pathogenic organisms.

Although acanthamoeba is a human pathogen (Khan, 2006; Binesh *et al.*, 2011; Coskun *et al.*, 2013; Inkinen *et.al.*, 2019) and it would have proved an additional worry if these would have been confirmed. However, the objective of the study was not to look for pathogenic amoebae alone but to investigate the role of FLA to protect pathogens from the normal treatment process and to act as a vehicle for these bacteria to survive the traditional wastewater treatment process. In other studies, as reviewed by Thomas and Ashbolt (2011) six other genera of FLA were identified that could act as hosts for ARB's and many of them presents with round cysts as were isolated in the majority of the samples tested during this study (Inkinen *et.al.*, 2019). Taking the results from this study and corroborates the reviews of Loret and Greub (2010), Thomas and Ashbolt (2011) and Vaerewijck *et.al.,*(2014) it is clear that there is a serious threat to human health if the presence of FLA is ignored (Dobrowsky, *et.al.*, 2017).

Looking at the distribution of the different types of cysts and amoeba in our study, unlike other reports (Valster *et. al*., 2009; Hsu, 2016), we found them throughout the year in all samples. We however noticed that in winter and spring only trophozoites and round cyst were found (Muchesa, *et al.,* 2014). Samples from the summer yielded similar results with one exception where one specimen had both presumptive *acanthamoeba* cysts and round cysts. However, all other presumptive *acanthamoeba* cysts were found in samples collected during the autumn sampling run. It is also during autumn that some of the samples only presented with cysts and no trophozoites (7 samples: 3 with presumptive *acanthamoeba* and 4 with round cysts). Furthermore, of the 30 samples that tested positive for intracellular bacteria, 24 samples were taken during the autumn sampling run. It would seem that the amoeba activity during the year stays quite constant but that of the *acanthamoeba* presence could be seasonal (Rodriquez-Zaragoza *et al,* 2005, Hsu, 2016; Inkinen *et.al.*, 2019). However, during the autumn sample run the plant were undergoing routine maintenance that could have released these amoeba and cysts from the biofilms present within these treatment units. This phenomenon is not uncommon as seen from a review by Thomas and Ashbolt (2011) where they describe breakthrough events in treatment plants and correlates with results found in a study done by Kao *et.al,* (2013) in which they observe more amoeba in the summer when the rains disturb the biofilm (Dobrowsky, *et.al.*, 2017; Inkinen *et.al.*, 2019). Furthermore, it is well documented that FLA's frequency are associated and present in biofilms (Lau and Ashbolt, 2009; Loret and Greub 2010; Dobrowsky, *et.al.*, 2017; Buse *et*

*al.,*2019; Inkinen *et al.*, 2019) that would be present in all water treatment processes, which could influence the outcome of monitoring these organisms because they are not in the sampled water but in the biofilm that does not form part of the sample. Thomas *et al.* (2010) states that suspended bacteria does not provide favourable grazing conditions for FLA's but that the optimal grazing conditions are only found in biofilms with which FLA's are integrally associated with (Bonilla-Lemus *et al*, 2013; Liu, 2018).

4.1.4 Amoeba Resistant Bacteria

In present study 17.4% (30 out of 172 samples) of all the samples had FLA, their cysts, and intracellular bacteria present. Of the 12.8% presumptive acanthamoeba positive samples (22 out of 172 samples) 90.9% (20 of 22 samples) presented with intracellular bacteria present. All 22 samples cultured were positive for *Escherichia coli* and S*higella* and 8 for *Mycobaterium avium* with only one of these samples being positive for *Salmonella*. Although round cysts were the most common found during this study only a few of these and the trophozoites associated with them presented with intracellular bacteria (5.8%). Furthermore, amoeba with intracellular bacteria in either trophozoites or cysts was found in all stages of the treatment process.

The samples (5.8%) that presented with round cysts and harboured intracellular bacteria. cultured positive for *E Coli* (5.8%), *Shigella* (5.8%), *Salmonella* (0.6%), *M avium* (3.5%) and *Legionella* (0.6%). The *Mycobacterium* found inside the round cysts were not identified as *Mycobacterium avium* through the PCR methods. Whereas the specimen that tested positive through culture and PCR for *Legionella* were the specimen that also tested positive for Acanthamoeba via PCR which was miss identified through microscopy as a sample with only round cysts.

Out of the 4 bacteria under investigation that could lead to diarrhoea, the number one killer disease of the young, the elderly and immune-compromised patient in Africa, 2 of these bacteria were found as part of the intracellular bacteria cultured during this study, namely *Escherichia coli* and *Shigella.*

4.1.4.1 Escherichia coli

In concurrence with literature, *Escherichia coli* isolates were the most abundant intracellular organisms found (Sibille *et al*., 1998; Thomas *et al*., 2010; Lambrecht *et.al.,*2015; Waso, *et.al.,* 2017). As it was present in all the samples and found intracellular (17.4%) as well as extra cellular (82.6%) to the cysts in all processes. Wherever amoeba with intracellular bacteria was found, *E Coli* was one of the many organisms cultured from them. The fact that this organism was found in all processes and that it survived all the treatment processes is an alarming concern. It was further shown in this study that part of the reason for this is, the fact that the organism could survive in the amoeba and thereby utilise their encystment process to protect it for the treatment protocol which is concurrent with current literature (Yousuf *et.al.,*2013; Lambrecht *et.al.,*2015). Although the specific pathotype of *Escherichia coli* was not determined by this study, it does show that if a strain like O157 were present in the sewage it could survive and cause infection (Okeke, 2009; Gimenez *et al.,* 2011; Chekabab *et al.*, 2013; Lambrecht *et.al.,*2015). Amoeba cysts have been known to survive up to 10 days and with it whatever has been internalised. In the current study, 26.2% of samples that were taken from the process just before the effluent leaves the plant showed the presence of intracellular *Escherichia coli*. According to Alsam *et al.* (2006) pathogenic strains of *Escherichia coli* showed a greater ability to survive in amoeba than non-pathogenic strains (Greub and Raoult, 2004; Yousuf *et al.*, 2013; Waso, *et.al.*, 2017), making our findings in this study even more troublesome.

4.1.4.2 Shigella

None of the environmental studies referenced from literature (Greub and Raoult, 2004; Alsam *et al.*, 2006; Thomas *et. al*., 2010, Thompson, Duy and Baker 2015) has found *shigella* as part of the range of ARB's. However, Jeong *et.al.,* (2007) proved with experimental procedures that amoeba can harbour S*higella sonnei* and suggested that this could be linked to several outbreaks in Korea. Furthermore Saeed *et.al.,* (2009, 2012) and Amir *et al.,* (2006) proved that both *Shigella sonnei* and *dysenteriae* can survive and multiply within FLA's (Thompson, Duy and Baker 2015). Unlike some environmental studies, our study reports that as with *Escherichia coli,* 99% of all the samples tested positive for *Shigella*. Twenty two percent of these positive samples were cultured from trophozoites and cysts with intracellular bacteria.

When we look at the seasonal distribution of our positive *shigella* isolates from intracellular bacteria from the 30 samples that tested positive, 24 were collected during the autumn collection run, only 2 samples each from the winter and spring runs and the summer collection. As with *Escherichia coli* positive intracellular isolates were obtained in the final process before the effluent left the plant. However, all the positive samples for this step were collected during the autumn run.

4.1.4.3 Salmonella

Salmonella however only 13 samples of the 172 samples collected were positive for *salmonella.* Out of the 13 positives samples only 2 were isolated as intracellular bacteria indicating lessor resistance to amoebal digestion. The lack of *Salmonella* in the samples tested could be due to the fact that *Salmonella* is known to be internalised and then kill off the host (Tezcan-Merdol *et al*, 2004; Liu, Whitehouse and Li, 2018) amoeba and therefore not effectively utilise the protective effects provided by these host to the treatment protocols used in these plants. This is despite the reports of numerous authors that the resistance of *S. typhimurium* and other organisms were increased to free chlorine (Brandl *et al.*, 2005; Adiba *et al.*., 2010; Bozzaro and Eichinger, 2011; Bridier *et al.*, 2011; Douesnard-Malo and Daigle, 2011; Denoncourt *et al.*, 2014; Liu, Whitehouse and Li 2018).

4.1.4.4 Mycobaterium Avium

As reported by Thomas *et al.* (2006) all the samples that cultured positive for *Mycobaterium sp* were isolated from amoeba or cysts or both (97/172) (Samba-Louaka *et al.,* 2018; Gebert *et al*., 2018). However, 14% (14/97) of these samples showed intracellular bacteria and *Mycobacteria* were cultured from these samples. *Mycobacterium sp.* had no preference to the type of amoeba or cysts it colonised as the number of samples that cultured positive from both round and *acanthamoeba* cysts were 6.2 and 8.3% respectively (Claeys and Robinson, 2018). Although the 14.5% of samples that demonstrated intracellular bacteria and were positive for *Mycobacterium* none of these could be confirmed as *Mycobacterium avium* through PCR. Nonetheless 1 sample that was confirmed as being *Mycobacterium avium* by PCR were cultured from a specimen where only extracellular bacteria were observed (Chern *et al.,* 2015). One of the intracellular samples that cultured positive for *Mycobacterium sp.* was found in the final process and once again like with *Escherichia coli* and *Shigella* this specimen was collected during the autumn collection. These positive cultures however were

collected during all four seasons. This study only focused on *Mycobacterium avium* as the *mycobacterium* of choice but according to Thomas *et al*., (2006) other environmentally waterborne *mycobacterium* could be opportunistic pathogens and therefore it is highly recommended further studies into the identification of the *mycobacterium* found in this study should be undertaken to rule out these strains of mycobacterium (Tran and Han, 2014; Chern *et.al.,* 2015). Taking this into consideration the fact that *mycobacterium* was present yearlong from the exit process of this plant is worrying as many of the *mycobacterium* sp can be opportunistic pathogens in immune-compromised patients (van der Wielen and van der Kooij, 2013; Claeys and Robinson 2018).

4.1.4.5 Legionella

Although *legionella* is the first most described ARB we did not find it extensively within this study. Only 7 positive samples of the 172 samples tested in this study, an additional 6 samples tested positive for *legionella* DNA through PCR. The only processes that did not test positive for the presence of *legionella* were the treated and untreated sewage. Of the 9 samples that tested positive for *legionella* only one of these was associated with intracellular bacteria and round cysts. Of the 7-positive sample. only 5 were collected during the summer run and 2 during the winter run, however those that were only positive for PCR were collected during the winter run. This strongly suggests that the bacterial numbers during the winter season could have been so low that it was unable to be cultured, or the bacteria were present but not viable for culture. Although the seasonal variation is a possibility the higher yield of PCR positive samples via PCR is well documented and reasons for this is the fastidious nature of the organism and that it is easily overgrown by other bacteria and damaged in the concentration steps of water analysis. Very often *Legionella* could be found in a viable but not culturable state in nature and with PCR only the DNA is replicated so it will also pick-up the DNA of non-viable bacteria (Yanez *et.al.,*2005; Velusamy *et. al*., 2010; Ceuppens *et.al.*, 2014; Ramamurthy *et al.*, 2014; Bentham and Whiley 2018).

4.1.4.6 Vibrio Cholera

Although *Vibrio cholera* is one of the more imperative diarrhoea causing bacteria, we only found one sample that tested positive for this organism during PCR, it tested negative for the DNA sequence used to identify *Vibrio cholera*. However, this same sample tested positive

for *Salmonella*, *Mycobaterium sp*., *Legionella* and *Escherichia coli* and was collected from the exit bioreactor effluent. This firstly raises the question as to where the *Vibro cholera* came from as it was not picked up during the rest of the sampling process and why there is such an number of pathogens still present in the bioreactor effluent leaving the plant. Further investigations into this are needed. Alam *et.al.* (2006) has described that *V. cholera* can go undetected during non-epidemic periods in a viable but non-culturable state. Furthermore, this author noted that *V cholera* is most often associated with biofilms and other aquatic organisms which protect them from detection (Lutz *et al.*, 2013; Conner *et. al*.,2016; Noorian *et. al*., 2017). Although the PCR methods utilised in this study failed to pick-up *Vibrio species* it might be advisable to expand the primer battery used to survey for *V cholera* and use a panel as was used by Alam *et.al.,* (2006).

4.1.4.7 Chlamydia

In our study we observed most of the samples that tested positive for *Chlamydia* through PCR have only extracellular bacteria present which was further associated with round cysts and amoebae trophozoites. This is in contrast with the literature that reports *Chlamydia* as a common intracellular bacterium and uses this phenomenon to isolate the organism (Collingro *et al.*, 2005). However, two of the samples that tested positive for *Chlamydia,* presented with intracellular bacteria and one with *acanthamoeba* cysts and the other with round cysts and amoeba trophozoites. In this study we further observed that there is an exceptionally low recovery rate for *Chlamydia* during the autumn sample run (9.1 %) whereas all the other seasons 50% or more of the samples tested positive (winter having 75%) for this bacterium. We could not find any reports of *Chlamydia* having seasonal variation. On the contrary Dumke *et.al,* (2015) reported no seasonal changes in *Chlamydia* infections in their study. Therefore, we either has stumbled on a new environmental factor governing the risk of *Chlamydia* infection from environmental sources or this might be due to artificial factors created by the study. One of such factors is that the plant was undergoing routine maintenance during the autumn sampling run and not all samples were used for PCR analysis and therefore we cannot with certainty say if the 9% positive rate is not underestimated. Whatever the true cause of this phenomenon is, it should be further investigated to fully understand and appreciate the risk factors related to the presence of *Chlamydia* in such high prevalence in wastewater treatment samples.

4.2 Conclusion

During our study we found that free living amoeba and their cysts were present throughout all seasons and all processes in the plant under investigation. Our hypothesis that acanthamoeba is the major FLA was not entirely correct as the FLA with round cyst were more abundant in the samples analysed. This study will recommend that a full study be undertaken to identify the FLA that occur in the wastewater treatment plants of Gauteng. This study further could not positively confirm the identity of the acanthamoeba identified via microscopy and further studies and other primers should be used as to identify the isolated FLA and their cysts. Although there are other factors from environmental samples that can interfere with the PCR techniques the fact that two of the samples tested positive for acanthamoeba rules those out (Schrader *et al.* 2012).

A further concern is the fact that acanthamoeba were only found in the one season except for one sample. Although according to literature (Rodriquez-Zaragoza *et al*, 2005) it could just be a seasonal effect that we observed (Hsu, 2016) or it could be a breakthrough event as described by Thomas and Ashbolt (2011) and others (Kao *et al*, 2013). The question is where they were hiding during the other seasons and where did they come from during the breakthrough event. This question could possibly be answered by the fact that they could be associated with biofilms as FLA's are more often found to colonise biofilms on surfaces of the plant and therefore analysis of just the water may be an under estimation of the problem and could explain why extracellular bacteria survived the different treatment stages (Thomas and Ashbolt, 2011; Buse *et.al.,*2019).

Numerous ARB's were found in the different samples analysed during this study and the most common for these were *E. coli* and *Shigella* that was present in almost all the samples tested. However, a shortcoming of this study was that the identification of these bacteria was based on selective media isolation and colony morphology which did not give us the sub species of *E. coli* and *Shigella* that was found. Although this was an exploratory study to prove the existence of these organisms in our treatment plants and their presence as ARB's further studies to further identify them to see which of the numerous *E. coli* and *Shigella* species are present here should be undertaken. The fact of their presence however should be alarming enough to put in place preventative measures and to include testing on a routine bases for these ARB's.

One of the objectives of this study was to assess the effectiveness of the current treatment process to remove FLA's and ARB's from the water. In our study the current treatment process has failed dismally to achieve this as our study showed that all samples collected throughout the year had some or all the organisms tested for in the final step of the plant. Furthermore, samples from the anaerobic section of the plant showed no reduction of trophozoites or bacteria as one would have thought it would. The only bacteria not recovered from this process were *legionella* and *V. Cholera*, however both were present in the final process step of the plant. Therefore, a serious re-visit to the processes and techniques currently used should be undertaken. The only specimen that was positive for *V. cholera* was collected from the effluent from this plant.

Another weakness in this study was that the autumn sampling run took place during routine maintenance and it was this season that was the only Acanthamoeba tested positive and very few of the samples tested positive for *Chlamydia sp*.

The effect of this maintenance should be established in a follow-up study.

4.2.1 Recommendations

Follow-up studies should be commissioned to address the following issues identified from this study:

- The effect of the maintenance on the prevalence of FLA's and ARB's (Specially *Chlamydia*.
- Taking not only water samples but also analysing the flora (FLA's and ARB's) of the biofilms in the different process if present.
- Further studies of different plants need to be undertaken to establish if this phenomenon is a global problem or just restricted to this plant.
- Extensive Molecular studies need to be undertaken to establish and identify the FLA's and ARB's (*Escherichia coli* and *Shigella, Mycobacterium*) to strain level as to ascertain the risk of possible human infections.
- Methods should be developed to reduce the transfer of FLA's and ARB between the different processes in the plant and to eliminate or reduce the development of biofilms.

- Furthermore, the PCR samples should be selected for positive cultured samples for the individual organisms cultured and not only on samples that cultured positive for *mycobacterium*. Further primers for other species of mycobacterium should be considered, as in this study very few of the cultured *mycobacterium sp.* turned out to be *mycobacterium avium* and there is other *mycobacterium sp.* that could be pathogenic in immune-compromised individuals in the community.

REFERENCES

A

Abd, H., Saeed, A., Weitraub, A., and Sandstron, G. 2009a Vibrio cholerae O139 requires neither capsule nor LPS O side chain to grow inside Acanthamoeba castellanii, *J. Med. Microbiol*, **58**, 125–131**.**

Abd H, Saeed A, Jalal S, Bekassy AN, Sandström G. 2009b Ante mortem diagnosis of amoebic encephalitis in a haematopoietic stem cell transplanted patient. *Scand J Infect Dis.;***41**(8),619-22.

Abd H, Saeed A, Weintraub A, Nair GB, Sandstrom G, 2007 Vibrio cholerae O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba Acanthamoeba castellanii. *FEMS Microbiol Ecol*, **60**(1)**,**33-39.

Abd H, Valeru Soni Priya, Sami Susan Marouf, Saeed Amir, Raychaudhuri Saumya,[3] and Sandström Gunnar 2010 Interaction between *Vibrio mimicus* and *Acanthamoeba castellanii Environ Microbiol Rep.* **2**(1), 166–171

Abd, H., Johansson, T., Golovliov, I., Sandström, G., and Forsman, M. 2003 Survival and growth of Francisella tularensis in Acanthamoeba castellanii, *Appl. Environ. Microbiol.* **69**, 600–606.

Abd, H., Weintraub, A. and Sandström, G. 2005 Intracellular survival and replication of Vibrio cholerae O139 in aquatic free-living amoebae. *Appl. Environ. Microbiol.* **7**, 1003–1008.

Abdel-Raouf, N., Al-Homaidan, A.A.,and Ibraheem, I.B.M. 2012 Microalgae and wastewater treatment *Saudi J Biol Sci.* **19**, 257–275

Abelman, R.L., M'ikanatha, N.M., Figler, H.M. and Dudley, E.G. 2019 Use of whole genome sequencing in surveillance for antimicrobial-resistant Shigella sonnei infections acquired from domestic and international sources. *Microb Genom.* **5**(5) :e000270. doi: 10.1099/mgen.0.000270. Epub 2019 May 17.

Addis, M. and Sisay, D. 2015 A Review on Major Food Borne Bacterial Illnesses. *J Trop Dis* **3**: 176. doi:10.4176/2329891X.1000176

Adiba S, Nizak C, van Baalen M, Denamur E, Depaulis F 2010 From Grazing Resistance to Pathogenesis: The Coincidental Evolution of Virulence *Factors. PLoS ONE* **5**(8): e11882. doi:10.1371/journal.pone.0011882

Agensi, A., Tibyangye, J., Tamale, A., Agwu, E. and Amongi, C. 2019 Contamination Potentials of Household Water Handling and Storage Practices in Kirundo Subcounty, Kisoro District, Uganda. *Journal of Environmental and Public Health*, https://doi.org/10.1155/2019/7932193**.**

Alam, M., Sultana, M., Nair, G.B., Sack, R.B., Sack, D.A., Siddique, A.K., 2006 Toxigenic *Vibrio cholerae* in the aquatic environment of Mathbaria, Bangladesh. *Appl Envir Microbiol* **72,** 2849–2855.

Alli, O. A. T., Olusoga, O. D., Adedokun, S. A. and Ogundare, O. E. 2011 Isolation of *Legionella pneumophila* from surface and ground waters in Osogbo, Nigeria. *Afri. J. Micro. Res.*, **5**(18), 2779-2785.

Al-Rekabi, W. S. Qiang, H and.Qiang, W. W 2007 Improvements in Wastewater Treatment Technology. *Pak J. of Nutr* **6**(2): 104-110.

Alsam S, Jeong SR, Sissons J, Dudley R, Kim KS, Khan NA 2006, Escherichia coli interactions with Acanthamoeba, a symbiosis with environmental and clinical implications. *J Med Microbiol* **55** (6), 689-694.

Al-Quadan, T., Price, C.T. and Kwaik, Y.A. 2012 Exploitation of evolutionarily conserved amoeba and mammalian processes by Legionella. *Trends Microbiol*. **20**(6): 299–306

Amahmid, O., Asmama, S. and Bouhoum, K. 2002. Urban Wastewater Treatment in Stabilisation Ponds: Occurrence and Removal of Pathogens. *Urban Wat*, **4,** 255-262.

Amir, S., Hadi, A., Benjamin, E., and Gunner S. 2006 Acanthamoeba castellanii an environmental host for Shigella dysenteriae and *Shigella sonne*. *Arch Microbiol*, **191**, 83-88.

Anaerobic treatment procedure http://www.fao.org/docrep/t0551e/t0551e0k.gif (Accessed 31May 2011)

Aerobic treatment procedure http://www.fao.org/docrep/t0551e/t0551e0k.gif (Accessed 31May 2011)

Angenent LT, Kelley ST, St Amand A, Pace NR, Hernandez MT 2005 Molecular identification of potential pathogens in water and air of a hospital therapy pool. *Proc Natl Acad Sci U S A.* **102**(13) 4860-5.

Arar, A. 1988 Background to treatment and use of sewage effluent. Ch. 2, Treatment and Use of Sewage Effluent for Irrigation, M.B. Pescod and A. Arar (eds). Butterworths, Sevenoaks, Kent.

Asano T., Smith R.G. and Tchobanoglous G. 1985 Municipal wastewater: Treatment and reclaimed water characteristics. Irrigation with Reclaimed Municipal Wastewater - A Guidance Manual, G.S. Pettygrove and T. Asano (eds). Lewis Publishers Inc., Chelsea, Mississippi.

Ashbolt N.J. 2015 Environmental (Saprozoic) Pathogens of Engineered Water Systems: Understanding Their Ecology for Risk Assessmentand Management. *Pathogens* **4**, 390-405;

Axelsson-Olsson, D., Waldenstrom, J., Broman, T., Olsen, B., and Holmberg, M. 2005 Protozoan Acanthamoeba polyphaga as a potential reservoir for Campylobacter jejuni. *Appl. Environ. Microbiol.* **71**, 987–992.

B

Backer, H. 2002 Water disinfection for international and wilderness travelers.*Clin. Infect. Dis*. **34**, 355–364.

Barbeau, J. and Buhler, T. 2001 Biofilms augment the number of free-living amoebae in dental unit waterlines. *Res Microbiol* **152**, 753–760.

Bardhan, P. Faruque, A.S.G. Naheed, A.and. Sack D. A. 2010 Decrease in Shigellosis-related Deaths without *Shigella spp*.–specific Interventions, Asia. *Emerg. Infect. Dis.* **16(**11*),* 1718-1723

Barratt, J. L. N., Harkness, J., Marriott, D., Ellis, J. T and Stark, D. 2010 Importance of Nonenteric Protozoan Infections in Immunocompromised People *Clinical Microbiology Reviews* **23** (4) 795-836; DOI: 10.1128/CMR.00001-10

Bartie, C., Klugman KP. 1997 Exposures to L pneumophila and C pneumoniae in South African mine workers. *Int. J. Occup. Environ. Health*; **3** (2), 120-127.

Bartie, C., Venter, SN,, Nel, LH. 2001 Chapter 56. Legionella detection from South African cooling water systems. In: Marre R, Abu Kwaik Y, Bartlett C, Cianciotto NP, Fields BS et al (eds). Legionella. ASM Press, Washington DC. 284-290. ISBN 1-55581-230-9.

Bartie C, Venter SN, Nel LH. 2003 Identification methods for Legionella from environmental samples. Water Res.; 37: 1362-1370.

Bateman, C. 2009 Cholera – Getting The Basics Right. , *SAMJ* **99**(3)

Bekale, R.B., Du Plessis, S.M., Hsu, N.J., Sharma, J.R., Sampson, S.L., Jacobs, M., Meyer, M., Morse, G.D. and Dube, A. 2018 Mycobacterium Tuberculosis and Interactions with the Host Immune System: Opportunities for Nanoparticle Based Immunotherapeutics and Vaccines. *Pharm Res.* ;**36**(1) 8. doi: 10.1007/s11095-018-2528-9.

Bentham, R. and Whiley, H. 2018 Quantitative Microbial Risk Assessment and Opportunist Waterborne Infections–Are There Too Many Gaps to Fill? *Int J Environ Res Public Health*. **15**(6): 1150. doi:10.3390/ijerph15061150

Berry, D., Horn, M., Xi, C., and Raskin,L. 2010, Mycobacterium avium Infections of Acanthamoeba Strains: Host StrainVariability, Grazing-Acquired Infections, and Altered Dynamics of Inactivation with Monochloramine *Appl Environ Microb*, **76**, (19), 6685–6688

Bichai, F., Payment, P. and Barbeau, B. 2008 Protection of waterborne pathogens by higher organisms in drinking water: a review, *Canadian J. of Microb.* **54,** 509-524.

Bichai, F., Barbeau, B. and Payment, P. 2009 Protection against UV disinfection of *Escherichia coli* bacteria and *B. subtilis* spores ingested by *C. elegans nematodes. Water Res.* **43**, 3397–3406

Binesh, F., Karimi, M. and Navabii, H. 2011 Unexpected Postmortem Diagnosis Of Acanthamoeba Meningoencephalitis In An Immunocompetent Child. *BMJ Case Reports* doi:10.1136/bcr.03.2011.3954

Black, A. D. 2008 The atypical pneumonias: the South African context. South Afr J *Epidemiol Infect* **23**(3), 34-38.

Bleasdale, B., Lott, P.J., Jagannathan, A. Stevens, M.P., Birtles, R. J. and Wigley, P. 2009 The Salmonella Pathogenicity Island 2-Encoded Type III Secretion System Is Essential for the Survival of *Salmonella enterica* Serovar *Typhimurium* in Free-Living Amoebae. *Appl Environ Microbiol.* **75**(6), 1793–1795

Bonilla-Lemus, P., Villegas, A. S. C.,. Jiménez, J. C. and. Vázquez, A. L. 2013 Occurrence of free-living amoebae in streams of the Mexico Basin. *Experimental Parasitology* **145,** S28–S33

Borodina, T. A, Lehrach, H., Soldatov, A. V. 2003 DNA purification on homemade silica spin-columns**,** *Anal. Biochem***. 321,** 135-137

Boyles, T.H., Brink, A., Calligaro, G.L., Cohen, C., Dheda, K., Maartens, G., Richards, G.A., van Zyl Smit, R., Smith, C., Wasserman, S., Whitelaw, A.C. and Feldman, C. 2017 South African Thoracic Society; Federation of Infectious Diseases Societies of Southern Africa. South African guideline for the management of community-acquired pneumonia in adults. *J Thorac Dis*. **9**(6)1469-1502. doi: 10.21037/jtd.2017.05.31. Erratum in: *J Thorac Dis.* 2018 **10**(8) E673-E675.

Bozzaro, S. and Eichinger, L. 2011 The Professional Phagocyte Dictyostelium discoideum as a Model Host for Bacterial Pathogens *Current Drug Targets***, 12,** 942-954

Brandl, M. T., Rosenthal, B. M., Haxo, A. F., and Berk, S. G. 2005. Enhanced survival of *Salmonella enterica* in vesicles released by a soilborne *Tetrahymena* species. *Appl. Environ. Microbiol*. **71**, 1562–1569.

Bridier, A., Briandet, R., Thomas, V. and Dubois-Brissonnet F. 2011 Resistance of bacterial biofilms to disinfectants: a review**.** *Biofouling: J Bioadhes. Biofilm Res.*, **27**(9), 1017-1032, DOI:10.1080/08927014.2011.626899

Brown, M.A., Potroz, M.G., Teh, S.W. and Cho, N.J. 2016 Natural Products for the Treatment of Chlamydiaceae Infections. *Microorganisms.* **4**(4) 39. doi: 10.3390/microorganisms4040039.

Bui, X. T., Wolff, A., Madsen, M. and Ban, D. D. 2012 Interaction between Food-borne Pathogens (Campylobacter jejuni, Salmonella Typhimurium and Listeria monocytogenes) and a Common Soil Flagellate (Cercomonas sp.) *J. Food Res.* **1**, (2); 76 - 81

Buse, H.Y., Morris, B.J., Struewing, I.T. and Szabo JG. 2019. Chlorine and monochloramine disinfection of Legionella pneumophila colonizing copper and polyvinyl chloride drinking water biofilms. *Appl Environ Microbiol* **85** e02956-18.

C

Cambridge dictionary **http://dictionary.cambridge.org/dictionary/british/sewage (checked 2020)**

Cardenal-Muñoz, E.. Barisch, C., Lefrançois, L.H., López-Jiménez, A.T. and Soldati, T 2018 When Dicty Met Myco, a (Not So) Romantic Story about One Amoeba and Its Intracellular Pathogen *Front. Cell. Infect. Microbiol.* | https://doi.org/10.3389/fcimb.2017.00529

Cervero-Aragó, S., Rodríguez-Martínez, S., Puertas-Bennasar, A. and Araujo, R.M. 2015 Effect of Common Drinking Water Disinfectants, Chlorine and Heat, on Free Legionella and Amoebae-Associated Legionella**.** ***PLoS ONE*** **10** (8)**:** e0134726. doi:10.1371/journal.pone.0134726

Ceuppens, S., Li D., Uyttendaele, M., Renault, P., Ross, P., Van Ranst, M., Cocolin, L. and Donaghy, J. 2014 Molecular Methods in Food Safety Microbiology: Interpretation and Implications of Nucleic Acid Detection. *Comprehensive Reviews in Food Science and Food Safety* **13**, 551-577

Chekabab S. M., Daigle, F., Charette, S. J., Dozois, C. M. and Harel J. 2013 Shiga toxins decrease enterohaemorrhagic Escherichia coli survival within Acanthamoeba castellanii. *FEMS Microbiol Lett.* **344,** 86–93

Chern, E.C., King, D., Haugland, R. and Pfaller, S. 2015 Evaluation of quantitative polymerase chain reaction assays targeting *Mycobacterium avium*, *M. intracellulare*, and *M. avium* subspecies paratuberculosis in drinking water biofilms. *J Water Health* **13** (1): 131–139. doi: https://doi.org/10.2166/wh.2014.060

Chikte UM, Khondowe O, Gildenhuys I; 2011 A case study of a dental receptionist diagnosed with legionnaires' disease; Department of interdisciplinary Health Sciences, Faculty of Health Sciences, Stellenbosch University , Tygerberg Campus, Parow, 7505,South Africa. *SADJ. **66** (6),284-7*

Cirillo J. D., Falkow S., Tompkins L S. And Bermudez L E. 1997 Interaction Of Mycobacterium Avium With Environmental Amoebae Enhances Virulence *Infec. Immun*. **65**(9), 3759–3767

Claeys, T.A. and Robinson, R.T. 2018 The many lives of nontuberculous mycobacteria. *J Bacteriol*. **200**(11) e00739-17. doi: 10.1128/JB.00739-17. [Epub ahead of print].

Clesceri, L. S. Greenberg, A. E. Eaton A. D. 1998 *Standard Methods for the Examination of Water and Wastewater*, 20th Edition APHA American Public Health Association

Collingro, A., S. Poppert, E. Heinz, S. Schmitz-Esser, A. Essig, M. Schweikert, M. Wagner, and M. Horn. 2005. Recovery of environmental chlamydia strain from activated sludge by co-cultivation with *Acanthamoeba* sp. *Microbiol.* **151**, 301-309.

Conner, J.G., Teschler, J.K., Jones, C.J. and Yildiz F.H. 2016 Staying Alive: Vibrio cholerae's Cycle of Environmental Survival, Transmission, and Dissemination. *Microbiol Spectr.* **4**(2) 10.1128/microbiolspec.VMBF-0015-2015. doi: 10.1128/microbiolspec.VMBF-0015-2015.

Conza, L, Pagani, S.C, Gaia, V. 2013 Presence of Legionella and Free-Living Amoebae in Composts and Bioaerosols from Composting Facilities. *PLoS ONE* **8**(7): e68244. doi:10.1371/journal.pone.0068244

Corbett EL, Blumberg L, Churchyard GJ, Moloi N, Mallory K, Clayton T, Williams BG, Chaisson RE, Hayes RJ, De Cock KM; 1999(a) Nontuberculous mycobacteria: defining disease in a prospective cohort of South Africa miners; Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom. *J Respir Crit Care Med;* **160**(1), 15-21.

Corbett EL, Churchyard GJ, Hay M, Herselman P, Clayton T, Williams B, Hayes R, Mulder D, De Cock KM.; 1999(b) The impact of HIV infection on Mycobacterium Kansasii disease in South Africa gold miners; Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom. *J Respir Crit Care Med*; **160**(1), 10-4.

Corsaro, D., Saucedo, G., Catalan, V., Loret, J.F., Greub, G., 2010. Biodiversity of amoebae and amoeba-associated bacteria in water treatment plants. *Int. J. Hyg. Environ. Health* **213** (3), 796–803

Coskun, K.A., Özçelik, S., Tutar, L., Elaldi, N., andTutar, Y. 2013 Isolation and Identification of Free-Living Amoebae from Tap Water in Sivas, Turkey. *BioMed Research International,* **2013,** 1-8

Coulon C , Collignon, McDonnell G, and Thomas V; 2010 Resistance of Acanthamoeba Cysts to Disinfection Treatment Used in Health care Settings *J. Clin. Microbiol., **48**(8),2689*.

Cronjé, F., Reyneke, S. and Van Wyk, D., 2013, 'Local communities and health disaster management in the mining sector', *Jàmbá: Journal of Disaster Risk Studies* **5**(2) Art. #78, 12 pages.http://dx.doi.org/10.4102/jamba.v5i2.78

Crowther-Gibson, P., Govender, N., Lewis, D.A., Bamford, C., Brink A., von Gottberg A.,, Klugman, K., du Plessis, M., Fali, A., Harris, B., Keddy, K. H. and Botha M. 2011 Part IV. Human infections and antibiotic resistance. *South African Medical Journal,* **101**, (8)

Croxatto, A., Murset, V., Chassot B. and Greub, G. 2013 Early expression of the type III secretion system of Parachlamydia acanthamoebae during a replicative cycle within its natural host cell Acanthamoeba castellanii. *Pathog. Dis.* , **69**, 159–175

Crump J. luby s and Mintz E. 2004 The global burden of typhoid fever; Bulletin of the world Health Organization; **82**, 346-353.

D

Das, S., Saha, R., Rani, M., Goyal, R., Shah, D. and. Asish, J.K 2016 Central nervous system infection due to Acanthamoeba: A case series. *Trop Parasitol.* **6**(1): 88–91.

Delafont, V.; Mougari, F.; Cambau, E.; Joyeux, M.;Bouchon, D.; Héchard, Y.; Laurent, M. 2014 First evidence of Amoebae−Mycobacteria association in drinking water network. *Environ. Sci. Technol.*, **48** , 11872−11882.

Dean, R.B. and Lund, E. 1981. *Water Reuse: Problems and Solutions.* Academic Press, New York.

Denoncourt, A. M., Paquet, V. E., and Charette, S. J. 2014 Potential role of bacteria packaging by protozoa in the persistence and transmission of pathogenic bacteria. *Front. Microbiol: Micro. Phys. Metabol.*. **5,** 1-10

Department of Water Affairs and Forestry, 1996. *South African Water Quality Guidelines (second edition)*, **1-8**

Deschamps, P. 2014 Primary endosymbiosis: have cyanobacteria and Chlamydiae ever been roommates? *Acta Soc Bot Pol* **83**(4), 291–302

Dewan, A.M., Corner, R., Hashizume, M. and Ongee, E.T. 2013 Typhoid Fever and Its Association with Environmental Factors in the Dhaka Metropolitan Area of Bangladesh: A Spatial and Time-Series Approach. *PLoS Negl Trop Dis* **7**(1): e1998. doi:10.1371/journal.pntd.0001998

Dini LA, Cockinos C, Frean JA, Niszl IA, Markus MB, 2000 Unusual case of Acanthamoeba polyphaga and Pseudomonas aeruginosa keratitis in a contact lens wearer from Gauteng, South Africa. *J Clin Microbiol.* **38**(2), 826-9.

Dlamini SK, Mendellson M; 2012 Atypical pneumonia in adults in southern Africa, Division of Infectious Diseases and HIV Medicine, Department of Medicine, University of Cape Town, Groote Schuur Hospital, Cape Town; *South Afr J Epidemiol.Infect.;***27**(1), 5-9

Dobrowsky, P. H. , De Kwaadsteniet, M.,. Cloete, and Khan,**W.**2014 Distribution of Indigenous Bacterial Pathogens and Potential Pathogens Associated with Roof-Harvested Rainwater *Applied and Environmental Microbiology* **80** (7), 2307–2316

Dobrowsky, P., Khan, S., Cloete, T., and Wesaal, K. 2017. Microbial and Physico-chemical Characteristics Associated with the Incidence of Legionella spp. and Acanthamoeba spp. in Rainwater Harvested from Different Roofing Materials. *Water, Air, & Soil Pollution*. **228**. 10.1007/s11270-017-3270-3.

Dobrowsky, P., Khan, S. and Wesaal, K. 2017. Resistance of Legionella and Acanthamoeba mauritaniensis to heat treatment as determined by relative and quantitative polymerase chain reactions. *Environmental Research*. **158**. 82-93. 10.1016/j.envres.2017.06.003.

Douesnard-Malo, F. and Daigle, F. 2011 Increased Persistence of *Salmonella enterica* Serovar *Typhi* in the Presence of *Acanthamoeba Castellanii*. *Appl. Environ. Microbiol.* **77**(21), 7640–7646

Dumke, R., Schnee, C., Pletz, M.W., Rupp, J., Jacobs, E., Sachse, K., Rohde, G. and CAPNETZ Study Group 2015 Mycoplasma pneumoniae and Chlamydia spp. Infection in Community-Acquired Pneumonia, Germany, 2011–2012. *Emerging Infect. Dis.* • **21**, (3), 426-434

E

Echeverria-Valencia, G., Flores-Villalva, S. and Espitia C.I. 2017. Virulence Factors and Pathogenicity of Mycobacterium, Mycobacterium - *Research and Development, Wellman Ribón, IntechOpen,* DOI: 10.5772/intechopen.72027. Available from: https://www.intechopen.com/books/mycobacterium-research-and-development/virulence-factors-and-pathogenicity-of-mycobacterium

Eddyani, M., De Jonckheere, J. F., Dumez, L., Suykerbuyk, P., Leirs, H., and Portaels F. 2008 Occurrence of free living amoebae in Communities of low and high endemicity for Buruli ulcer in Southern Benin, *Appl. Environ. Microbiol.*, **74** (21), 6547-6553.

Ekwanzala, M.D., Abia, A.L.K, Keshri, J., and Momba, M.N.B. 2017 Genetic characterization of Salmonella and Shigella spp. isolates recovered from water and riverbed sediment of the Apies River, South Africa. *Water SA,* **43**(3), 387-397. https://dx.doi.org/10.4314/wsa.v43i3.03

El-Etr, S. H., Margolis, J. J., Monack, D., Robison, R. A., Cohen, M., Moore, E. and Rasley, A. 2009 Francisella tularensis Type A Strains Cause the Rapid Encystment of Acanthamoeba castellanii and Survive in Amoebal Cysts for Three Weeks Postinfection. *Appl. Environ. Microbiol.*, **75**(23). 7488–7500

Emch, M., Feldacker ,C., Sirajul, I.M. and Ali, M. 2008 Seasonality of cholera from 1974 to 2005: a review of global patterns. *Int J Health Geo*. **7**. 31

Environmental-expert: http://www.environmental-expert.com/services/wastewater-definition-of-117681 (checked 2020)

Estrada-Garcia, T., Lopez-Saucedo, C., Thompson-Bonilla, R, Abonce, M., Lopez-Hernandez, D., Santos, J. I., Rosado, J. L. DuPont, H.L. and Long, K.Z. 2009 Association of Diarrheagenic Escherichia coli Pathotypes with Infection and Diarrhea among Mexican Children and Association of Atypical Enteropathogenic E. coli with Acute Diarrhea, *J. Clin. Microbiol*. **47**(1) 93–98

Everett, K. D. E.; Bush, R. M.; Andersen, A. A. 1999. "Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards for the identification of organisms". *Int. J. Syst. Bacteriol.***49** (2), 415–440

F

Falkinham, J.O., Pruden, A. and Edwards, M. 2015 Opportunistic Premise Plumbing Pathogens: Increasingly Important Pathogens in Drinking Water. *Pathogens.* **4**(2) 373-86. doi: 10.3390/pathogens4020373. PMID: 26066311; PMCID: PMC4493479.

Farnleitner AH, Ryzinska-Paier G, Reischer HG, Burtscher MM, Knetsch S, Kirschner AKT, Dirnbock T, Kuschnig G, Mach RL, and Sommer R; 2010 Escherichia coli and enterococci are sensitive and reliable indicators for human, livestock and wildlife faecal pollution in alpine mountainous water resources**.** *J. Appl. Microbiol.* **109,** 1599–1608**.**

Faruque SM and Nair GB. 2008. *Vibrio cholerae*: Genomics and molecular biology. Caister Academic Press, UK.

Flemming, H. and Wuertz, S. 2019 Bacteria and archaea on Earth and their abundance in biofilms. *Nat Rev Microbiol.* **17,** 247–260, doi:10.1038/s41579-019-0158-9

Fouque, E., Trouilhé, M., Thomas, V., Hartemann, P., Rodier, M. and Hécharda, Y. 2012 Cellular, Biochemical, and Molecular Changes during Encystment of Free-Living Amoebae. *Eukaryotic Cell*,**11** (4), 382–387

Friedman, M. G., Kahane, S., Dvoskin, B. and. Hartley, J. W 2006 Detection of Simkania negevensis by culture, PCR, and serology in respiratory tract infection in Cornwall, UK. *J Clin Pathol.* **59**, 331–333.

Fukumoto, T., Matsuo, J., Hayashi, M., Oguri, S., Nakamura, S., Mizutani, Y., Yao, T., Akizawa, K., Suzuki, H., Shimizu, C., Matsuno, K., and Yamaguchi, H. 2010 Impact of Free-Living Amoebae on Presence of Parachlamydia acanthamoebae in the Hospital Environment and Its Survival In Vitro without Requirement for Amoebae. *J Clin Microbiol* **48**(9) 3360–3365

G

Galván-Moroyoqui JM, Del Carmen Domínguez-Robles M, Franco E, Meza I. 2008 The interplay between Entamoeba and enteropathogenic bacteria modulates epithelial cell damage. *PLoS Negl Trop Dis*.; **2**(7), 266.

Garcia, A., Goñi, P., Cieloszyk, J., Fernandez, M.T., Calvo-Beguería, L., Rubio, E., Fillat, M. F., Peleato, M.L. and Clavel, A. 2013 Identification of Free-Living Amoebae and Amoeba-Associated Bacteria from Reservoirs and Water Treatment Plants by Molecular Techniques. *Environ. Sci. Technol*., **47** (7), 3132–3140

Gaze, W.H., Burroughs, N., Gallagher, M.P., and Wellington, E.M. 2003 Interactions between Salmonella typhimurium and Acanthamoeba polyphaga, and observation of a new mode of intracellular growth within contractile vacuoles, *Microb. Ecol*. **46**, 358–369.

Gebert, M. J., Delgado-Baquerizo, M., Oliverio, A.M.,Webster, T.M., Lauren M. Nichols, L.M., Honda, J.R. Chan, E.D.,Adjemian, J.,Dunn, R.R. and Fierer, N. 2018 Ecological Analyses of Mycobacteria in Showerhead Biofilms and Their Relevance to Human Health *mBio.* **9**(5) DOI: 10.1128/mBio.01614-18

George, I., Crop, P. & Servais, P. 2002. Fecal Coliform Removal in Wastewater Treatment Plants Studied by Plate Counts and Enzymatic Methods. *Wat. Res*. **36**, 2607-2617

Gião, M.S., Azevedo, N.F., Wilks, S.A., Vieira, M.J. and Keevil, C.W. 2011 Interaction of Legionella pneumophila andhelicobacter pylori with bacterial species isolated from drinking water biofilms. *BMC Microbiol.* **11**, 57

Gimenez, G., Bertelli, C., Moliner, C., Robert, C., Roult D., Fournier E. P. and Greub, G.: 2011 Insight into cross-talk between intra-amoebal pathogens; unite des rickettsies. *BMC Genomics* **12**, 542

Gomes, T.S., Gjiknuri, J., Magnet, A., Vaccaro, L., Ollero, D., Izquierdo, F., Fenoy, S., Hurtado, C. and del Águila, C. 2018 The Influence of Acanthamoeba–Legionella Interaction in the Virulence of Two Different Legionella Species. *Front. Microbiol*. **9** 2962. doi: 10.3389/fmicb.2018.02962

Goñi, P., Fernándezm, M.T. and Rubio, E. 2014 Identifying Endosymbiont Bacteria Associated With Free-Living Amoebae. *Environ. Microbiol.* **16**(2), 339–349

Grant, S R.; Shashi G., James, C. Y.; Robert L.; Albert C. C.; Churn C. C. 2002 An Anaerobic Exercise: A comparison of anaerobic treatment technologies for industrial wastewater. *Environ Protect.* **Nov,** 18–26

Greub, G. 2009 Parachlamydia acanthamoebae, an emerging agent of pneumonia. *Clin Microbiol Infect* **15**, 18–28

Greub G and Raoult D 2003 Rhodobacter massiliensis sp. nov., a new amoebae-resistant species isolated from the nose of a patient. *Res Microbiol* **154**, 631–635.

Greub G, Berger P, Papazian L, Raoult D. 2003 Parachlamydiaceae as rare agents of pneumonia*. Emerg Inf. Dis*.; **9** (6), 755-756.

Greub, G., and Raoult, D. 2004 Microorganisms resistant to free-living amoebae. *Clin. Microbiol. Rev.*, **17** (2), 413-433.

Greub, G., B. La Scola, and D. Raoult. 2004. Amoebae-resisting bacteria isolated from human nasal swabs by amoebal coculture. *Emerg*. *Infect. Dis*. **10**, 470-477.

Gryseels, S., Amissah, D., Durnez, L., Vandelannoote, K., Leirs, H., De Jonckheere, J., Silva, M.T., Portaels, F., Ablordey, A. and Eddyani, M., 2012 Amoebae as Potential Environmental Hosts for Mycobacterium ulcerans and Other Mycobacteria, but Doubtful Actors in Buruli Ulcer Epidemiology. *PLoS Negl Trop Dis* **6**(8): e1764. doi:10.1371/journal.pntd.0001764

H

Hamilton, K., Reyneke, B., Waso, M., Clements, T., Ndlovu,T., Khan, W., DiGiovanni, K., Rakestraw, E., Montalto, F.,Haas, C.N. and Ahmed, W. 2019 A global review of the microbiological quality and potential health risks associated with roof-harvested rainwater tanks.*npj Clean Water* **2,** 7 doi:10.1038/s41545-019-0030-5

Harrison, K. 2011 Types of Diseases caused by E. coli. *Home Diseases and Health Conditions Infections Escherichia coli*: http://www.livestrong.com/article/132628-types-diseases-caused-e-coli. last updated May 2011. Last visited Jan 2014

Hillman P.J. 1988 *Health aspects of reuse of treated wastewater for irrigation. Ch. 5, Treatment and Use of Sewage Effluent for Irrigation.* M.B. Pescod and A. Arar (eds). Butterworths, Sevenoaks, Kent**.**

Hsu, B., Huang, C., Chen, J., Chen, N. and Huang, J. 2011 Comparison of potentially pathogenic free-living amoeba hosts by Legionella spp. in substrate-associated biofilms and floating biofilms from spring environments. *Water Research* **45,** 5171 - 5183

Hsu, B. 2016 Surveillance and Evaluation of the Infection Risk of Free-Living Amoebae — Acanthamoeba in Aquatic Environments. *IJESD*, **7**, (6), 445-448

Huws SA, Smith AW, Enright MC, Wood PJ, Brown MRW. 2006 Amoebae promote persistence of epidemic strains of MRSA. *Environ Microbiol,* **8** (6), 1130 - 1133.

Huws, S. A., Morley, R. J., Jones, M. V, Brown, M. R, W, and Smith, A. W. 2008 Interactions of some common pathogenic bacteria with Acanthamoeba polyphaga. *FEMS Microbiol Lett*, **282**, 258-262.

I

Inkinen, J., Jayaprakash, B., Siponen, S., Hokajärvi, A., Pursiainen, A., Ikonen. J., Ryzhikov, I., Täubel, M., Kauppinen, A., Paananen, J., Miettinen, I.T., Torvinen, E., Kolehmainen, M., and Pitkänen, T. 2019 Active eukaryotes in drinking water distribution systems of ground and surface waterworks. *Microbiome***, 7,** 99 doi:10.1186/s40168-019-0715-5

Iperepolu OH, Entonu PE, Agwale SM. 2008 A review of the disease burden, impact and prevention of typhoid fever in Nigeria. *West Afr J Med*. **27**(3), 127-33.

Isberg, R. R., O'Connor, T. J. and Heidtman, M.. 2009. The Legionella pneumophila replication vacuole: making a cosy niche inside host cells. *Nature Rev. Microbiol* **7**(1): 13-24

J

Jadin, J.B. 1975 Amibes Limax vecteurs possibles de Mycobacteries et de M. leprae. *Acta Leprol,* 59, 57–67.

Janda, W.M. 2010 Amoeba-Resistant Bacteria: Their Role in Human Infections, *Clinical Microbiology Newsletter* **32,** (23), 177-184.

Jeong, H.J., Jang, E.S., Han, B.I., Lee, K.H., Ock, M.S., Kong, H.H., Chung, D.I., Seol, SY, Cho DT, Yu HS: 2007 Acanthamoeba: could it be an environmental host of Shigella? *Exp Parasitol*, **115**(2)**,** 181-186.

Ji, W.. Hsu, B., Chang, T., Hsu, T., Kaoa, P., Huang, K., Tsai, S., Huang, Y. and Fan, C. 2014 Surveillance and evaluation of the infection risk of free-living amoebae and Legionella in different aquatic environments. *Sci. Total Environ.* **499**, 212–219

Jjemba, P. K., Weinrich, L. A., Cheng, W., Giraldo, E, and LeChevallier, M. W. 2010 Regrowth of Potential Opportunistic Pathogens and Algae in Reclaimed-Water Distribution Systems. *Appl Environ Microbiol***, 76**(13), 4169–4178

K

Kabwama, S.N., Bulage, L., Nsubuga, F., Pande, G., Oguttu, D.W., Mafigiri, R., Kihembo, C., Kwesiga, B., Masiira, B., Okullo, A.E., Kajumbula, H., Matovu, J.K.B., Makumbi, I., Wetaka, M., Kasozi, S., Kyazze, S., Dahlke, M., Hughes, P., Sendagala, J.N., Musenero, M., Nabukenya, I., Hill, V.R., Mintz, E., Routh, J., Gómez, G., Bicknese, A., Zhu, B.P. 2017 A large and persistent outbreak of typhoid fever caused by consuming contaminated water and street-vended beverages: Kampala, Uganda, January – June 2015. *BMC Public Health* **17**(1):23. doi: 10.1186/s12889-016-4002-0.

Kalule, J.B., Smith, A.M., Vulindhlu, M., Tau, N.P., Nicol, M.P., Keddy, K.H. and Robberts, L. 2019 Prevalence and antibiotic susceptibility patterns of enteric bacterial pathogens in human and non-human sources in an urban informal settlement in Cape Town, South Africa. BMC Microbiol. **19**(1) 244. doi: 10.1186/s12866-019-1620-6.

Kao, P., Chou, M., Tao, C., Huang, W., Hsu, B., Shen, S., Fan, C. and Chiu, Y. 2013 Diversity and Seasonal Impact of Acanthamoeba Species in a Subtropical Rivershed. *BioMed Research International* **2013**, Article ID 405794, 8 pages http://dx.doi.org/10.1155/2013/405794

Kariuki S. 2008 Typhoid fever in sub-Saharan Africa: challenges of diagnosis and management of infections. *J Infect Dev Ctries.* **2**(6), 443-7.

Karstaedt AS; 2012 Profile of cause of death assigned to adults on antiretroviral therapy in Soweto. *S Afr. Med. J.,* **102**(8), 680-682

Khattak, F.A., Kumar, A., Kamal, E., Kunisch, R. and Lewin, A. 2012 Illegitimate recombination: An efficient method for random mutagenesis in

Mycobacterium avium subsp. Hominissuis. *BMC Microbiology*, **12**, 204 http://www.biomedcentral.com/1471-2180/12/204

Kebbi-Beghdadi, C. and Greub, G. 2014 Importance of amoebae as a tool to isolate amoeba-resisting microorganisms and for their ecology and evolution: the *Chlamydia* paradigm. *Environmental Microbiology Reports.* doi: 10.1111/1758-2229.12155

Keddy KH, Sooka A, Crowther-Gibson P, Quan V, Meiring S, Cohen C, Nana T, Sriruttan C., Seetharam S, Hoosen A, Naicker P, Elliott E, Haffejee S, Whitelaw A, Klugman Kp; 2012 for the Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa (GERMS-SA) Systemic shigellosis in South Africa. *Clin Infect Dis; **54**(10); 1448-1454*.

Keddy KH; 2010 *Communicable Diseases Surveillance Bulletin **8***(2)

Keddy KH; 2011 *Communicable Diseases Surveillance Bulletin* **9**(*2)*

Keddy KH; 2012 *Communicable Diseases Surveillance Bulletin* **10**(3)

Keddy, K.H., Smith, A.M., Sooka, A., Tau, N.P., Ngomane, H.M.P., Radhakrishnan, A., Als, D. and Benson, F.G. 2018 The Burden of Typhoid Fever in South Africa: The Potential Impact of Selected Interventions. *Am J Trop Med Hyg.* **99**(3_Suppl):55-63. doi: 10.4269/ajtmh.18-0182. Epub 2018 Jul 24.

Keddy K. H., Sooka A., Ismail H., Smith A. M., Weber I., Letsoalo M. E. And Harris B. N. 2011 Molecular epidemiological investigation of a typhoid fever outbreak in South Africa, 2005: the relationship to a previous epidemic in 1993. *Epidemiol. Infect.* **139**(08),1239-1245

Khan, N. A. 2006 Acanthamoeba :Biology And Increasing Importance In Human Health. *FEMS Microbiol Rev* **30,** 564–595

Khan, M.A., Knox, N., Prashar, A., Alexander, D., Abdel-Nour M, Duncan, C., Tang, P., Amatullah, H., Dos Santos, C.C., Tijet, N., Low, D.E., Pourcel, C., Van Domselaar, G., Terebiznik, M., Ensminger, A.W. and Guyard, C., 2013 Comparative Genomics Reveal That Host-Innate Immune Responses Influence the Clinical Prevalence of Legionella pneumophila Serogroups. *PLoS ONE* **8**(6): e67298. doi:10.1371/journal.pone.0067298

Kicka, S., Trofimov, V., Harrison, C., Ouertatani-Sakouhi, H., McKinney, J., Scapozza, L., Hilbi, H., Cosson, P. and Soldati, T. 2014 Establishment and Validation of Whole-Cell Based Fluorescence Assays to Identify Anti-Mycobacterial Compounds Using the *Acanthamoeba castellanii - Mycobacterium marinum* Host-Pathogen System. *PLoS ONE* **9**(1): e87834.doi:10.1371/journal.pone.0087834

King CH, Shotts EB, Wooley RE, Porter KG, 1988 Survival of coliforms and bacterial pathogens within protozoa during chlorination. ***Appl Environ Microbiol*****,** 54**(12)**, **3023-3033.**

Kinge W. C. , Mbewe M.; 2010 Characterisation of Shigella Species Isolated from River Catchments in The North West Province of South Africa. S Afr J Sci.; 106(11/12), 211-4

Koestler, B.J., Ward, C.M., Fisher, C.R., Rajan, A., Maresso, A.W. and Payne, S.M. 2019 Human Intestinal Enteroids as a Model System of Shigella Pathogenesis**.** *Infect Immun.* **87**(4) :e00733-18. doi: 10.1128/IAI.00733-18.

Koh Pei Xiu, Chiou Shun Chien, Ajam Noni, Watanabe Haruo, Ahmad Norazah and Thong Lin Kwai; 2012 Characterization of Shigella sonnei in Malaysia, an

increasingly prevation etiologic agent of local shigellosis cases, *BMC Infec. Dis.* **12**, 122-129.

Kokashvili, T., Whitehouse, C.A., Tskhvediani, A., Grim, C.J., Elbakidze, T., Mitaishvili, N., Janelidze, N., Jaiani, E., Haley, B.J., Lashkhi, N., Huq, A., Colwell, R.R., and Tediashvili, M. 2015 Occurrence and Diversity of Clinically Important Vibrio Species in the Aquatic Environment of Georgia. *Front Public Health*. **13**(3) 232. doi: 10.3389/fpubh.2015.00232.

Kothari, A., Pruthi, A. and Chugh, T. D. 2008 The Burden of Enteric Fever. *J Infect Developing Countries* **2**(4): 253-259.

Krishna-Prasad, B. N., and Gupta, S. K.. 1978. Preliminary report on engulfment and retention of mycobacteria by trophozoites of axenically grown *Acanthamoeba castellanii* Douglas.*Curr. Sci.***47,** 245-247.

Kustner, H. G. V. and Du Plessis, G. 1991. The cholera epidemic in South Africa, 1980 – 1987., SAMJ 79, 539

L

Lamoth, F. and Greub, G., 2010. Amoebal pathogens as emerging causal agents of pneumonia. *FEMS Microbiol. Rev*. **34**, 260–280.

Lambrecht, E., Baré, J., Chavatte, N., Bert, W., Sabbe, K., Houf, K. 2015. Protozoan cysts act as a survival niche and protective shelter for foodborne pathogenicbacteria. *Appl Environ Microbiol* **81**, 5604 –5612. doi:10.1128/AEM.01031-15.

Lamrabet, O., Medie, F.M. and Drancourt, M. 2012 Acanthamoeba polyphaga-Enhanced Growth of *Mycobacterium smegmatis. PLoS ONE* **7**(1), e29833. doi:10.1371/journal.pone.0029833

Lau, H. Y.; Ashbolt, N. J. 2009 The role of biofilms and protozoa in *Legionella* pathogenesis: Implications for drinking water. *J. Appl. Microbiol.*, **207**(2), 368–378.

Le, P.T., Ramulu, H.G., Guijarro, L., Paganini, J., Gouret, P., Chabrol, O., Raoult, D. and Pontarotti, P. 2012 An automated approach for the identification of horizontal gene transfers from complete genomes reveals the rhizome of *Rickettsiales. BMC Evolutionary Biology* **12**:243, http://www.biomedcentral.com/1471-2148/12/243

Loret, J-F., and Greub, G. 2010 Free-living amoebae: biological by-passes in water treatment. *Int J Hyg Envir Heal,* **213**(3), 157-232.

Liu, H., Whitehouse, C. and Li, B. 2018. Presence and Persistence of Salmonella in Water: The Impact on Microbial Quality of Water and Food Safety. *Frontiers in Public Health*. **6**. 10.3389/fpubh.2018.00159.

Liu, W. 2018. Synergistic Interactions within a Multispecies Biofilm Enhance Individual Species Protection against Grazing by a Pelagic Protozoan. *Frontiers in Microbiology*. **8**. 10.3389/fmicb.2017.02649.

Lutz, C., Erken, M., Noorian, P., Sun, S. and McDougald, D. 2013 Environmental reservoirs and mechanisms of persistence of *Vibrio cholerae Front. Microbiol.: Aqua.Microbiol.* **4,** 1-15

M

MacLean RC, Hafez N, Tripathi S, Childress CG, Ghatak NR, Marciano-Cabral F. 2007 Identification of Acanthamoeba sp. in paraffin-embedded CNS tissue from an HIV+ individual by PCR. *Diagn Microbiol Infect Dis*. **57**(3), 289-94.

Marciano-Cabral, F. and Cabral, G. A. 2007 The Immuneresponse To *Naegleria Fowleri* Amebae and Pathogenesis of Infection. *FEMS Immunol Med Microbiol.* **51**, 243–259

Marciano-Cabral, F., Jamerson, M. and Kaneshiro, E. S. 2010 Free-living amoebae, Legionella and Mycobacterium in tap water supplied by a municipal drinking water utility in the USA. *J. Water and Health* **08**(1), 71-82

Mari L, Bertuzzo E, Righetto L, Casagrandi R, Gatto M, Rodriguez-Iturbe I, Rinaldo A. 2012 Modelling cholera epidemics: the role of waterways, human mobility and sanitation. *J R Soc Interface.* **9**(67), 376-88.

Mba Medie, F., Ben Salah, I., Henrissat, B., Raoult, D. and Drancourt, M. 2011 *Mycobacterium tuberculosis* Complex Mycobacteria as Amoeba-Resistant Organisms. *PLoS ONE* **6**(6), e20499. doi:10.1371/journal.pone.0020499

Mbuthia, O.W., Mathenge, S.G., Oyaro, M.O. and Ng'ayo, M.O. 2018 Etiology and pathogenicity of bacterial isolates: a cross sectional study among diarrheal children below five years in central regions of Kenya. *Pan Afr Med J.* **4**(31) 88. doi: 10.11604/pamj.2018.31.88.15644.

Mella, C., Medinab, G., FloresMartina, S., Toledoc, Z., Simaluizac, R.J., PérezPérezd, G., Fernándeza, H., 2016 Interaction between zoonotic bacteria and free living amoebas. A new angle of an epidemiological polyhedron of public health importance? *Arch. med. vet.* **48** (1) 1-10

Mercante, J.W. and Winchell, J.M. 2015 Current and Emerging Legionella Diagnostics for Laboratory and Outbreak Investigations. *Clin. Microbiol. Rev.,* **28** (1). 95-133

Metcalf and Eddy. 2003. Wastewater Engineering: Treatment and Reuse. McGraw-*Microbiol. Rev.* **34**(3), 231-259.

Mogoa E, Bodet C, Morel F, Rodier MH, Legube B, Héchard Y. 2011 Cellular response of the amoeba Acanthamoeba castellanii to chlorine, chlorine dioxide, and monochloramine treatments. *Appl Environ Microbiol.* **77**(14),4974-80.

Mohamed, M.E., Huseein, E.A., Farrag, H.M., Mostafa, F.A.A. and Hassan, A.T. 2016 *Allovahlkampfia spelaea* is a Potential Environmental Host for Pathogenic Bacteria. *J Bacteriol Parasitol* **7**: 255. doi:10.4172/2155-9597.1000255

Moliner C, Fournier PE, Raoult D. 2010 Genome analysis of microorganisms living in amoebae reveals a melting pot of evolution. *FEMS Microbiol Rev***. 34**(3), 281-94

Molino, P., Bentham, R., Higgins, M., Hinds, J and Whiley, H. 2019. Public Health Risks Associated with Heavy Metal and Microbial Contamination of Drinking Water in Australia. *International Journal of Environmental Research and Public Health*. **16**. 3982. 10.3390/ijerph16203982.

Molmeret, M., Horn, M., Wagner, M., Santic, M. and Abu Kwaik, Y. 2005 Amoebae as training grounds for intracellular bacterial pathogens. *Appl Environ Microb* **71**, 20–28.

Monack, D.M., Mueller, A. and Falkow, S. 2004 Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nat. Rev. Microbiol.* **2**(9):747–765.

Morris, J. G. Jr. 2013 Minor-vibrio-and-vibrio-like-species-associated-with-human-disease, http://www.uptodate.com/contents/minor-vibrio-and-vibrio-like-species-associated-with-human-disease updated 2013.

Mpenyana-Monyatsi, L., Onyango, M.S., Momba, M.N.B. 2012 Groundwater Quality in a South African Rural Community: A Possible Threat to Public Health. *Pol. J. Environ. Stud*. **21**, (5) 1349-1358

Muchesa P, Barnard TG, Bartie C. 2015 The prevalence of free-living amoebae in a South African hospitalwater distribution system. *S Afr J Sci*.,**111**(1/2), Art. #2014-0278, 3 pages. http://dx.doi.org/10.17159/sajs.2015/20140278

Muchesa, P., Mwamba, O., Barnard T. G., and Bartie, C. 2014 Detection of Free-Living Amoebae Using Amoebal Enrichment in a Wastewater Treatment Plant of Gauteng Province, South Africa, *BioMed Research International*, **2014**, https://doi.org/10.1155/2014/575297.

N

Niyyati, M., Mafi, M., Haghighi, A. and Hakemi Vala M., 2015 Occurrence of Potentially Pathogenic Bacterial-Endosymbionts in Acanthamoeba Spp. *Iran J Parasitol* **10**, (2), 181-188

Noorian, P., Hu, J., Chen, Z., Kjelleberg, S., Wilkins, M.R., Sun, S. and McDougald, D. 2017 Pyomelanin produced by Vibrio cholerae confers resistance to predation by Acanthamoeba castellanii. *FEMS Microbiol Ecol*. **93**(12) fix147. doi: 10.1093/femsec/fix147. PMID: 29095994; PMCID: PMC5812506.

O

Odonkor, S.T. and Ampofo J.K. 2013 Escherichia coli as an indicator of bacteriological quality of water: an overview. *Microbiology Research*; **4**(e2), 5-11

Oguntoke O, Aboderin OJ, Bankole AM. 2009 Association of water-borne diseases morbidity pattern and water quality in parts of Ibadan City, Nigeria. *Tanzan J Health Res*. ;**11**(4),189-95.

Okaiyeto, K., Nwodo, U.U., Okoli, S.A., Mabinya, L.V. and Okoh, A.I. 2016 Implications for public health demands alternatives to inorganic and synthetic flocculants: bioflocculants as important candidates. *Microbiologyopen*. **5**(2):177-211. doi: 10.1002/mbo3.334.

Okeke, I.N. 2009 Diarrheagenic Escherichia coli in sub-Saharan Africa: status, uncertainties and necessities *J Infect Dev Ctries.* **3**(11), 817-42

Olofsson, J., Axelsson-Olsson, D., Brudin, L., Olsen B., and Ellström, P. 2013 *Campylobacter jejuni* Actively Invades the Amoeba *Acanthamoeba polyphaga* and Survives within Non Digestive Vacuoles. *PLoS ONE* **8**(11): e78873. doi:10.1371/journal.pone.0078873

Olofsson, J., Griekspoor Berglund, P., Olsen, B., Ellström, P., and Axelsson-Olsson, D. 2015 The abundant free-living amoeba, *Acanthamoeba polyphaga*, increases the survival of Campylobacter jejuni in milk and orange juice. *Infection Ecology and Epidemiology* **5**: 28675 - http://dx.doi.org/10.3402/iee.v5.28675

Omsland, A., Sixt, B.S., Horn, M. and Hackstadt, T. 2014 Chlamydial metabolism revisited: interspecies metabolic variability and developmental stage-specific physiologic activities. *FEMS Microbiol Rev,* **38,** 779–801

Ovrutsky, A.R., Chan, E.D., Kartalija, M., Bai, X., Jackson, M., Gibbs, S., Falkinham III, J.O., Iseman, M.D., Reynolds, P.R., McDonnell, G., and Thomas, V. 2013 Cooccurrence of Free-Living Amoebae and Nontuberculous Mycobacteria in Hospital Water Networks, and Preferential Growth of *Mycobacterium avium* in *Acanthamoeba lenticulata. Appl EnvironMicrob.* **79**(10), 3185–3192

P

Pagnier, I., Merchat, M., La Scola, B. 2009 Potentially pathogenic amoeba-associated microorganisms in cooling towers and their control. *Future Microbiol*. **4**(5), 615-629.

Pagnier, I., Roult, D. and La Scola, B. 2008 Isolation and identification of amoeba-resisting bacteria from water in human environment by using Acanthamoeba Polyphaga co-culture procedure. *Environ. Microbiol.* **10** (5), 1135-1144.

Percival, S.L., Chalmers, R.M., Embrey, M., Hunter, P.R., Sellwood, J., Wyn-Jones, P. 2004: Legionella. In: Microbiology of Waterborne Diseases, first ed. *Elsevier Academic Press, California*, USA, 145–153.

Perez-Martinez, I., Aguilar-Ayala, D.A., Fernandez-Rendon, E., Carrillo-Sanchez1, A.K., Helguera-Repetto, A.C., Rivera-Gutierrez, S., Estrada-Garcia, T., Cerna-Cortes, J.F., and Gonzalez-y-Merchand J.A. 2013 Occurrence of potentiallypathogenic nontuberculous mycobacteria in Mexican household potable water: a pilot study. *BMC Res. Notes* **6**, 531. http://www.biomedcentral.com/1756-0500/6/531

Perilla, M.J. 2003 Manual for the laboratory identification and antimicrobial testing of bacterial pathogens of public health importance in the developing world. World Health Organization. *Atlanta Georges U.S.A*, 133 –284.

Phin, N., McLean, E., Naik, F.C., Zhao, H. and Harrison, T. 2012 Legionnaires' disease in England and Wales, 2008-2010. *Health Protection Report* **6** (9)

Potroz, M.G. and Cho, N. 2015 Natural Products for the Treatment of Trachoma and Chlamydia trachomatis. *Molecules,* **20**, 4180-4203; doi:10.3390/molecules20034180

Potts, A., Donaghy, M., Marley, M., Othieno, R., Stevenson, J., Hyland, J., Pollock, K.G., Lindsay, D., Edwards, G., Hanson, M,F. and Helgason, K.O. 2013 Cluster of Legionnaires' disease cases caused by *Legionella longbeachae serogroup 1*, Scotland, August to September 2013 . Euro Surveill. **18**(50):pii=20656. Available online: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20656

Q

Qurie, M., Khamis, M., Scrano, L., Bufo S.A. and Karaman R. 2015 Toward Zero Liquid Discharge from Integrated Advanced Wastewater Treatment Plant. *Case Stud. J.* **4** (6), 64-77

R

Ramamurthy, T., Ghosh, A., Pazhani, G.P. and Shinoda, S. 2014 Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. *Front. Public Health,* **2,** doi: 10.3389/fpubh.2014.00103

Reddy R, Vijayasaradhi M, Uppin MS, Challa S, Jabeen A, Borghain R. 2011 Acanthamoeba meningoencephalitis in an immunocompetent patient: an autopsy case report. *Neuropath.*. **31**(2),183-7.

Riquelme, S., Varas, M., Valenzuela, C., Velozo, P., Chahin, N., Aguilera, P., Sabag, A., Labra, B., Álvarez, S.A., Chávez, F.P. and Santiviago, C.A. 2016 Relevant Genes Linked to Virulence Are Required for Salmonella Typhimurium to

Survive Intracellularly in the Social Amoeba Dictyostelium discoideum. *Front. Microbiol.* **7**:1305. doi: 10.3389/fmicb.2016.01305

Rodriquez-Zaragoza S, Mayzlish E and Steinberger Y 2005 Shrubs in the Negev Desert, Israel Amoeba Population in Soil under Desert Vertical Distribution of the Free-Living *Appl. Environ. Microbiol*., **71**(4), 2053.

Rojas-Lopez, M., Monterio, R., Pizza, M., Desvaux, M. and Rosini, R. 2018 Intestinal Pathogenic Escherichia coli: Insights for Vaccine Development, Front. Microbiol., **20** https://doi.org/10.3389/fmicb.2018.00440

Romalde, J.L., Diéguez, A.L., Lasa A. and Balboa, S. 2014 New Vibrio Species Associated To Molluscan Microbiota: A Review. *Front.Microbiol* **4,** doi: 10.3389/fmicb.2013.00413

Rompré, A., Servais, P., Baudert, J., de-Roubin, M. and Laurent, P. 2002. Detection and Enumeration of Coliforms in Drinking Water: Current Methods and Emerging Approaches. *J. Micro. Meth*. **49**, 31-54.

Rowbotham, T. J. 1983 Isolation of *Legionella pneumophila* from clinical samples via amoebae, and the interaction of those and other isolates with amoebae. *J. Clin. Pathol* **33**, 1179-1183.

Rubenièa, I., Kirjuðina, M., Bçrziòð, A., Valcièa, O. and Jahundovièa, I. 2017 Relationships between free-living amoeba and their intracellular bacteria. *Proceedings of The Latvian Academy Of Sciences. Section B*, **71**, 4 259–265.

Ryan, K. J., Ray, C. G. 2004 *Sherris Medical Microbiology* (4th ed.). McGraw Hill. ISBN 0-8385-8529-9.

S

Saeed A, Johansson D, Sandström G, Abd H. 2012 Temperature Depended Role of Shigella flexneri Invasion Plasmid on the Interaction with Acanthamoeba castellanii. *Int J Microbiol*.; **2012,** Article 917031.

Saeed, A., Abd, H., Edvinsson, and B., Sandström, G. 2009 Acanthamoeba castellanii an environmental host for Shigella dysenteriae and Shigella sonnei. *Arch. Microbiol*, **191**, 83–88.

Sakran, T., Matooq, P., El-Shahawy,G., Shalaby, M., Sabry, H. and Gad, M. 2019 Monitoring Water Quality Parameters in Egyptian Tap Water. Pol. J. Environ. Stud. **28**(4) 2815-2821

Samba-Louaka, A., Robino, E., Cochard, T., Branger, M., Delafont, V., Aucher, W., Wambeke, W., Bannantine, J.P., Biet, F., and Héchard, Y. 2018 Environmental *Mycobacterium avium* subsp. *paratuberculosis* Hosted by Free-Living Amoebae. *Front. Cell. Infect. Microbiol*. **8** 28. doi: 10.3389/fcimb.2018.00028

Samhan, F.A., Kronlein, M.R., Fakher, U., Kronlein, C., Stedtfeld, R.D. and Hashsham, S.A. 2015 Detection and Occurrence of Indicator Organisms and Pathogens. *Water Environ. Res.*, **87**(10) 883-900

SANS 241-1, 2015. Drinking Water Specification, Edition 2. South African National Standard Drinking water. Pretoria.

SANAC 2017 National strategic plan for HIV,TB and STI's 2017-2022 https://sanac.org.za//wp-content/uploads/2017/06/NSP_FullDocument_FINAL.pdf

Sandström G, Saeed A, Abd H. 2010 Acanthamoeba polyphaga is a possible host for Vibrio cholerae in aquatic environments. *Exp Parasitol*. **126**(1), 65-68.

Scheid, P 2014 Relevance of free-living amoebae as hosts for phylogenetically diverse microorganisms. *Parasitol. Res.*, **113**, (7), 2407-2414

Schuster FL, Visvesvara GS 2004 Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int J Parasitol.* **34**(9), 1001-27.

Schuster FL. 2002 Cultivation of pathogenic and opportunistic free-living amoeba. *Clin. Microbiol. Rev.*; **15**(3), 342-354.

Schrader, C., Schielke, A., Ellerbroek, L. and Johne, R. 2012 PCR inhibitors – occurrence, properties and removal. *J Appl Microbiol,* **113:** 1014-1026. https://doi.org/10.1111/j.1365-2672.2012.05384.x

Sengupta, C. and Saha, R. 2013 Understanding coliforms - a short review *Int. J. of Adv. Res.* **1**(4) 16-25

Senkbeil, J.K., Ahmed, W., Conrad, J. and Harwood, V.J. 2019 Use of Escherichia coli genes associated with human sewage to track fecal contamination source in subtropical waters. *Science of The Total Environment* **686**, 1069-1075

September S.M, Brozel V.S, and Venter S.N; 2004 Diversity of Nontuberculoid Mycobacterium Species in Biofilms of Urban and semiurban Drinking Water Distribution Systems; *App. Environ. Microbiol,* **70**(12), 7571-7573.

Shanan, S., Bayoumi, M., Saeed, A., Sandström, G.,and Abd, H., 2016 Swedish isolates of *Vibrio cholerae* enhance their survival when interacted intracellularly with *Acanthamoeba castellanii. Infec. Ecol. Epidemiol.* **6,** http://dx.doi.org/10.3402/iee.v6.31060

Sibille, I., Sime-Ngando, T., Mathieu, L. and Block J. C. 1998 Protozoan Bacterivory and *Escherichia coli* Survival in Drinking Water Distribution Systems. *Appl. Environ. Microbiol.*, **641** 197–202.

Siddiqui, R., Malik, H., Sagheer, M., Jung, S. and. Khan, N. A. 2011 The type III secretion system is involved in *Escherichia coli* K1 interactions with *Acanthamoeba. Exp. Parasitol.* **128,** 409–413

Singh, T. and Coogan, MM. 2005 Isolation of pathogenic Legionella specie and Legionella-laden amoebae in dental unit waterlines. *Hosp infect;* **61**(3), 257-62.

Singh,T.S. and Matuka, O. 2013 Work-related infections –Part 1: Risks of exposure to infectious agents in the workplace. *Occupational Health Southern Africa.* **19**(2)

Snelling, W. J., Moore, J. E., McKenna, J. P., Lecky, D. M. and Dooley, J. S. G. 2006 Bacterial protozoa interactions; an update on the role these phenomena play towards human illness. *Microbes and Infec.,* **8**, 578–587.

Schmitz-Esser, S., Toenshoff, E. R., Haider, S., Heinz, E.,. Hoenninger, V. M, Wagner, M. and Horn M. 2008 Diversity of Bacterial Endosymbionts of Environmental Acanthamoeba Isolates *App Environ Microb*, **74**(18) 5822–5831

Srinu, N. S. and Pydi, S.Y. 2011 Biological Denitrification Of Wastewater In A Fluidized Bed Bioreactor By Immobilization Of Pseudomonas Stutzeri Using Poly Propylene Granules. *Int J of Biot Appl* **3**(3), 106-109

Steinberg, KM., Levin, BR. 2007 Grazing protozoa and the evolution of the Escherichia coli 0157:h7 Shiga toxin-encoding prophage. *Proc Royal Soc;* **274**, 1921-1929.

Statistics South Africa 2014 Statistical release P0211 Quarterly Labour Force Survey, Quarter 4, http://www.statssa.gov.za/publications/P0211/P02114thQuarter2014.pdf

Strebel PM, Ramos JM, Eidelman IJ, Tobiansky L, Koornhof HJ, Kustner HG; 1988 Legionnaire' disease in Johannesburg teaching hospital. Investigation and control of outbreak; Department of National Health and Population Development, Pretoria *S Afr Med J.*; **73**(6), 329-33

T

Tezcan-Merdol, D., Ljungstrom, M., Winiecka-Krusnell, J., Linder, E., Engstrand, L. *and* Rhen, M. 2004 Uptake and replication of *Salmonella enterica* in *Acanthamoeba rhysodes. Appl Environ Microbiol* **70***,* 3706–3714.

Tecon, R. and Or, D., 2017 Biophysical processes supporting the diversity of microbial life in soil, *FEMS Microbiol Rev*. **41(5),** 599–623.

Thomas, V., Loret, JF., Jousset, M. & Greub, G. 2008 Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant. *Environ Microbiol* **10**, 2728–2745.

Thomas, J. M and Ashbolt, N. J. 2011 Do Free-Living Amoebae in Treated Drinking Water Systems Present an Emerging Health Risk? *Environ. Sci. Technol.,* **45** (3), 860-869.

Thomas, V. and McDonnell, G. 2007 Relationship between mycobacteria and amoebae: ecological and epidemiological concerns, *Lett. Appl. Microbiol* **45**, 349–357.

Thomas, V., Herrera-Rimann, K., Blanc, D. S., and Greub, G. 2006 Biodiversity of amoebae and amoeba-resisting bacteria in a Hospital water network, *Appl. Environ. Microbiol.*, **72**(4) 2428-2438.

Thomas, V., McDonnel, G., Denyer, S., Maillard, J.Y., 2010. Free-living amoebae and their intra-cellular pathogenic micro-organisms: risks for water quality. *FEMS Microbiol. Rev.* **34**(3), 231-259.

Thompson, F. L., Lida, T. and Swings, J. 2004 Biodiversity of vibrios. *Microbiol Mol Biol R,* **68**, 403–431.

Thompson, C.N., Duy, P.T. and Baker, S. 2015 The Rising Dominance of Shigella sonnei: An Intercontinental Shift in the Etiology of Bacillary Dysentery. *PLoS Negl Trop Dis* **9**(6), doi:10.1371/journal.pntd.0003708

Tobiansky, L., Drath, A., Dubery, B., Koornhof, H.J. 1986 Seasonality of Legionella isolates from environmental sources. *Isr J Med Sci.* **22**(9):640-3. PMID: 3793446.

Todar K 2007. "Pathogenic *Escherichia coli*". *Online Textbook of Bacteriology*. University of Wisconsin–Madison Department of Bacteriology

Tran, Q.T. and Han, X.Y. 2014 Subspecies Identification and Significance of 257 Clinical Strains of *Mycobacterium avium*. *J. Clin. Microbiol.* **52**(4) 1201–1206

U

Uribe-Querol, E. and Rosales, C. 2017 Control of Phagocytosis by Microbial Pathogens. *Front. Immunol*. https://doi.org/10.3389/fimmu.2017.01368

Utsev, J. T. and Agunwamba, J. C. 2012 The Effects Of Aspect Ratio On Solar Enhanced Waste Stabilization Ponds. *J Sci Tech*, **32**(1) 42-554

V

Vaerewijck, M.J.M., Baré, J., Lambrecht, E., Sabbe, K. and Houf, K. 2014 Interactions of Foodborne Pathogens with Free-living Protozoa: Potential Consequences for Food Safety. *Compr. Rev. Food Sci. Food Saf.* **13**, 924-944

Valster, R. M., Wullings, B. A., Bakker, G.Smidt, H. and van der Kooij, D. 2009 Free-Living Protozoa in Two Unchlorinated Drinking Water Supplies, Identified by Phylogenic Analysis of 18S rRNA Gene Sequences. *Appl. Environ. Microbiol.,*,**75** (14), 4736–4746.

Van Niekerk, AM. and Schneider, B. 2013 WRC Report: Implementation Plan for Direct and Indirect Water Re-use for Domestic Purposes – Sector Discussion Document, WRC Report No. KV 320/13, ISBN 978-1-4312-0484-7

van der Wielen, P.W. J. J. and van der Kooij, D. 2013 Nontuberculous Mycobacteria, Fungi, and Opportunistic Pathogens in Unchlorinated Drinking Water in the Netherlands. *Appl. Environ. Microbiol.***9**(3) 825–834

Velusamy, V., Arshak, K., Korostynska, O., Oliwa, K. and Adley, C. 2010 An Overview Of Foodborne Pathogen Detection: In The Perspective Of Biosensors. *Biotechnol. Adv.* **28,** 232–254

Visvesvara GS, Moura H, Schuster FL 2007 Pathogenic and opportunistic free-living amoebae: Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri, and Sappinia diploidea. *FEMS Immunol Med Microbiol.* **50**(1), 26.

Vubil, D., Acácio, S., Quintò, L., Ballesté-Delpierre, C., Nhampossa, T., Kotloff, K., Levine, M.M., Alonso, P., Nataro, J.P., Farag, T.H., Vila, J. and Mandomando, I. 2018 Clinical features, risk factors, and impact of antibiotic treatment of diarrhea caused by Shigella in children less than 5 years in Manhiça

District, rural Mozambique. *Infect Drug Resist.* **31(**11) 2095-2106. doi: 10.2147/IDR.S177579.

W

Wang H, Pryor MA, Edwards MA, Falkinham J.O. 3rd and Pruden A.. 2013 Effect of GAC pre-treatment and disinfectant on microbial community structure and opportunistic pathogen occurrence. *Water Res*. **47**(15):5760-72

Warren, B. R., Parish, M. U, & Schneider, K. R. 2006. Shigefla us a foodhorne pathogen and current *methods for* detection in food. *Crit RevFood Sri Nan* **46**, 551-557.

Waso, M., Dobrowsky, P., Hamilton, K., Puzon, G., Miller, H., Wesaal, K., and Ahmed, W.. 2017. Abundance of Naegleria fowleri in roof-harvested rainwater tank samples from two continents. *Environmental Science and Pollution Research*. **25.** 10.1007/s11356-017-0870-9.

Winiecka-Krusnell J, Wreiber K, von Euler A, Engstrand L & Linder E 2002 Free-living amoebae promote growth and survival of Helicobacter pylori. *Scand J Infect Dis* **34**, 253–256.

Wheat, W.H., Casali, A.L., Thomas, V., Spencer, J.S., Lahiri, R., Williams, D.L., McDonnell, G.E., Gonzalez-Juarrero, M., Brennan1, P.J. and Jackson, M. 2014 Long-term Survival and Virulence of *Mycobacterium leprae* in Amoebal Cysts. *PLoS Negl Trop Dis* **8**(12): e3405. doi:10.1371/journal.pntd.0003405

Wolter, N., Carrim, M., Cohen, C., Tempia, S., Walaza, S., Sahr, P., de Gouveia, L., Treurnicht, Fl., Hellferscee, O., Cohen, A.L., Benitez, A.J., Dawood, H., Variava, E., Winchell, J.M. and von Gottberg, A. 2016 Legionnaires' Disease in South Africa, 2012–2014. *Emerg. Infect. Dis.* **22**(1), 131-133

World Health Organisation: Regional office for Africa 2011 *Outbreak bulletin*, **1**(6).

World Health Organization 2002 Sustainable Development and Healthy Environments: Managing Water in the Home: Accelerated Health Gains from Improved Water Supply, Geneva.

World Water. 1987 Biological plant beats cold. 21-23.

Wu S, Kuschk P, Wiessner A, Kästner M, Pang C, Dong R. 2013 Response of removal rates to various organic carbon and ammonium loads in laboratory-scale constructed wetlands treating artificial wastewater *Water Environ Res*. **85**(1), 44-53.

Y

Yamane,C., Yamazaki, T., Nakamura, S., Matsuo, J., Ishida, K., Yamazaki, S., Oguri1, S., Shouji, N., Hayashi, Y., Yoshida M.,, Yimin, and Yamaguchi, H., 2015 Amoebal Endosymbiont *Parachlamydia acanthamoebae Bn9* Can Grow in Immortal Human Epithelial HEp-2 Cells at Low Temperature; An In Vitro Model System to Study Chlamydial Evolution. *PLoS ONE* **10**(2). doi:10.1371/journal.pone.0116486

Yanez, M., Carrasco-Serrano, A.C., Barbera, V.M., and Catala´ n, V. 2005 Quantitative detection of Legionella pneumophila in watersamples by immunomagnetic purification and real-time PCR amplification of the dotA gene. *Appl. Environ. Microbiol,* **71**(7), 3433–3441.

Yousuf, F.A., Siddiqui, R. and Khan N.A. 2013 *Acanthamoeba castellanii* of the T4 genotype is a potential environmental host for *Enterobacter aerogenes* and *Aeromonas hydrophila*. *Parasit Vectors* **6**, 169.

Yu, H.S., Jeong, H.J., Hong, Y.C., Seol, S.Y., Chung, D.I., Kong, H.H., 2007. Natural occurrenceof Mycobacterium as an endosymbiont of Acanthamoeba isolated from a contact lens storage case. *Korean J. Parasitol*. **45**, 11–18.

Z

Zanella, J, Pinto da Costa, S. O., Zacaria, **J. and Echeverrigaray**, **S.** 2012 A Rapid and Reliable Method for the Clonal Isolation of Acanthamoeba from Environmental Samples**.** *Braz. Arc. Biol. Tech.* **55**(1)1-6

Zusman T, Feldman M, Halperin E & Segal G 2004 Characterization of the icmH and icmF genes required for Legionella pneumophila intracellular growth, genes that are present in many bacteria associated with eukaryotic cells. *Infect Immun* **72**, 3398–3409.

Appendix A:

A1 Raw Data

Table A-1: Physio-chemical and Amoeba data.

Code	Sample Source	Season	Temperature	pH	Incubated	Trophozoites	A Cysts	Round Cysts	Comments
IM 1071	1	1	21,5	6,75	33	1	1	2	Trophozolites , intracellular bacteria in cysts.
IM 1073	1	1	20,2	6,98	33	1	1	2	Trophozolites , intracellular bacteria in cysts
IM 1103	1	1	18,4	6,33	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1104	1	1	22,9	7,36	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1072	1	1	21,7	7,11	33	2	1	2	Intracellular bacteria in cysts.
IM 1093	1	1	12,9	7,54	33	1	2	1	Trophozoites , intracellular bacteria in cysts
IM 1094	1	1	15,4	7,30	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1107	1	1	18,9	6,41	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1091	1	1	12,6	7,39	33	1	2	1	Trophozoites , extracellular bacteria
IM 1105	2	1	16,1	7,31	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1074	2	1	22,1	7,22	33	1	2	1	Trophozolites , intracellular bacteria in cysts
IM 1082	2	1	17,8	7,67	33	2	2	1	Extracellular bacteria intracelluar bacteria
IM 1096	3	1	19,5	7,33	33	1	2	1	Trophozoites , intracellular bacteria in cysts
IM 1075	3	1	22,1	7,19	33	2	1	2	Intracellular bacteria in cysts.
IM 1083	3	1	21,3	7,47	33	1	1	2	Trophozoites , intracellular bacteria in cysts
IM 1108	3	1	17,8	7,32	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1076	4	1	22,3	6,94	33	2	2	1	Extracellular bacteria intracelluar bacteria
IM 1113	4	1	18,7	7,12	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1084	4	1	18,1	6,81	33	1	1	2	Trophozoites , intracellular bacteria in cysts

Code	Sample Source	Season	Temperature	pH	Incubated	Trophozoites	A Cysts	Round Cysts	Comments
IM 1090	4	1	15,1	7,90	33	1	1	2	Trophozoites , intracellular bacteria in cysts
IM 1099	4	1	18,3	6,90	33	1	1	2	Trophozoites , intracellular bacteria in cysts
IM 1077	5	1	21,4	6,97	33	1	1	2	Trophozolites , intracellular bacteria in cysts
IM 1111	5	1	16,2	7,12	33	1	1	2	Trophozoites , intracellular bacteria in cysts
IM 1085	5	1	17,7	6,84	33	1	1	2	Trophozoites , intracellular bacteria in cysts
IM 1100	5	1	17,4	6,99	33	1	1	2	Trophozoites , intracellular bacteria in cysts
IM 1078	6	1	21,4	6,92	33	1	1	2	Trophozolites , intracellular bacteria in cysts
IM 1086	6	1	18,9	6,90	33	1	1	2	Trophozoites , intracellular bacteria in cysts
IM 1095	6	1	15,9	7,30	33	1	1	2	Trophozoites , extracellular bacteria cysts
IM 1112	6	1	16,2	7,12	33	1	2	1	Trophozoites , entracellular bacteria in cysts
IM 1087	7	1	16,1	7,16	33	1	1	2	Trophozoites , intracellular bacteria in cysts
IM 1097	7	1	16,5	7,24	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1079	7	1	20,7	6,97	33	2	2	1	Extracellular bacteria intracelluar bacteria
IM 1110	7	1	17,4	7,44	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1088	7	1	17,4	7,11	33	2	1	2	Intracellular bacteria in cysts
IM 1080	7	1	22,2	7,13	33	2	2	1	Extracellular bacteria intracelluar bacteria
IM 1098	7	1	15,4	7,80	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1109	7	1	13,1	7,33	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1106	8	1	16,8	7,42	33	1	1	2	Trophozoites , intracellular bacteria in cysts

Code	Sample Source	Season	Temperature	pH	Incubated	Trophozoites	A Cysts	Round Cysts	Comments
IM 1081	8	1	22,1	7,31	33	1	1	2	Trophozolites , intracellular bacteria in cysts.
IM 1089	8	1	17,8	7,89	33	1	1	2	Trophozoites , intracellular bacteria in cysts
IM 1092	8	1	16,4	7,34	33	1	1	2	Trophozoites , intracellular bacteria in cysts
IM 1167	1	2	12,4	7,54	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1159	1	2	7,7	8,13	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1160	1	2	7,5	7,70	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1165	1	2	13,2	7,71	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1200	1	2	11,8	7,26	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1187	1	2	11,1	7,55	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1188	1	2	8,9	7,50	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1201	1	2	11,6	7,27	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1158	1	2	6,6	7,66	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1164	1	2	10,5	7,61	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1191	1	2	8,6	7,64	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1202	1	2	11,5	7,33	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1189	2	2	14,6	7,41	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1151	2	2	10,9	7,28	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1197	3	2	16,5	7,29	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1208	3	2	12,1	7,27	33	1	2	1	Trophozoites , extracellular bacteria in cysts

Code	Sample Source	Season	Temperature	pH	Incubated	Trophozoites	A Cysts	Round Cysts	Comments
IM 1156	3	2	13,3	7,17	33	1	2	1	Trophozoites , intracellular bacteria in cysts
IM 1166	3	2	15,9	7,32	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1196	4	2	13,3	6,65	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1155	4	2	13,2	7,18	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1163	4	2	14,4	7,55	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1172	4	2	15,6	6,93	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1206	4	2	11,5	7,07	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1168	5	2	14,9	6,81	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1152	5	2	9,1	7,02	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1193	5	2	13,1	6,91	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1203	5	2	11,6	7,10	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1153	6	2	9,4	6,99	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1207	6	2	11,7	7,03	33	1	2	1	Trophozoites , entracellular bacteria in cysts
IM 1170	6	2	14,8	7,13	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1192	6	2	13,6	6,96	33	1	2	1	Trophozoites , entracellular bacteria in cysts
IM 1169	7	2	14,1	7,32	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1154	7	2	11,1	7,12	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1194	7	2	14,1	7,28	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1204	7	2	11,4	7,25	33	1	2	1	Trophozoites , extracellular bacteria in cysts

Code	Sample Source	Season	Temperature	pH	Incubated	Trophozoites	A Cysts	Round Cysts	Comments
IM 1205	7	2	12,6	7,51	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1195	7	2	15,6	7,06	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1157	7	2	8,9	7,20	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1171	7	2	16,1	6,91	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1162	8	2	13,8	7,73	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1199	8	2	13,4	7,55	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1150	8	2	15,2	7,22	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1190	8	2	18,2	7,12	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1231	1	3	22,0	7,30	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1255	1	3	20,8	7,16	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1256	1	3	19,1	7,01	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1220	1	3	16,5	7,42	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1234	1	3	21,1	7,33	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1243	1	3	22,6	7,25	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1244	1	3	22,0	7,38	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1258	1	3	18,8	6,67	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1221	1	3	20,6	7,42	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1223	1	3	16,9	7,40	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1232	1	3	20,5	7,36	33	1	2	1	Trophozoites , extracellular bacteria in cysts

Code	Sample Source	Season	Temperature	pH	Incubated	Trophozoites	A Cysts	Round Cysts	Comments
IM 1246	1	3	22,7	7,20	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1219	2	3	19,3	7,76	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1230	2	3	20,8	7,32	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1242	2	3	22,1	7,22	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1254	2	3	19,1	6,91	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1228	3	3	21,1	7,36	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1239	3	3	21,4	7,15	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1251	3	3	22,8	7,11	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1303	3	3	20,4	7,28	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1240	4	3	20,7	7,38	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1304	4	3	19,1	6,87	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1229	4	3	20,1	6,91	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1252	4	3	22,8	6,90	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1235	5	3	21,3	7,14	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1247	5	3	22,2	7,05	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1224	5	3	23,6	7,00	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1259	5	3	19,1	6,92	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1248	6	3	22,6	7,12	33	1	2	1	Trophozoites , entracellular bacteria in cysts
IM 1225	6	3	20,3	7,12	33	1	2	1	Trophozoites , entracellular bacteria in cysts

Code	Sample Source	Season	Temperature	pH	Incubated	Trophozoites	A Cysts	Round Cysts	Comments
IM 1236	6	3	20,2	7,26	33	1	2	1	Trophozoites , entracellular bacteria in cysts
IM 1300	6	3	20,7	7,07	33	1	2	1	Trophozoites , entracellular bacteria in cysts
IM 1226	7	3	20,2	7,31	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1227	7	3	20,2	7,39	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1302	7	3	19,9	7,16	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1237	7	3	21,2	7,33	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1238	7	3	12,7	7,28	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1249	7	3	22,5	7,27	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1250	7	3	22,8	7,10	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1301	7	3	19,0	7,08	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1245	8	3	22,8	7,20	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1233	8	3	21,4	7,31	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1257	8	3	20,2	7,25	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1222	8	3	18,5	7,42	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1555	1	4	23,2	7,24	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1556	1	4	22,3	7,36	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1572	1	4	27,7	7,26	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1573	1	4	26,3	7,42	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1588	1	4	25,7	7,15	33	1	2	1	Trophozoites , extracellular bacteria in cysts

Code	Sample Source	Season	Temperature	pH	Incubated	Trophozoites	A Cysts	Round Cysts	Comments
IM 1329	1	4	24,4	6,82	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1330	1	4	25,8	7,20	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1332	1	4	24,5	7,09	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1554	1	4	23,5	7,44	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1571	1	4	26,8	7,38	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1586	1	4	25,7	7,29	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1587	1	4	25,7	7,29	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1328	2	4	25,0	7,19	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1570	2	4	27,7	7,52	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1579	3	4	25,7	6,84	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1337	3	4	24,4	7,70	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1593	3	4	25,8	6,77	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1561	3	4	24,8	7,29	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1562	4	4	24,4	7,11	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1580	4	4	25,6	6,99	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1585	4	4	25,7	7,31	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1594	4	4	25,6	6,89	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1338	4	4	25,3	6,91	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1553	4	4	24,2	7,20	33	1	2	1	Trophozoites , extracellular bacteria in cysts

Code	Sample Source	Season	Temperature	pH	Incubated	Trophozoites	A Cysts	Round Cysts	Comments
IM 1575	5	4	27,6	7,12	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1333	5	4	26,0	6,99	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1557	5	4	25,5	7,18	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1589	5	4	25,7	6,84	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1590	6	4	25,7	7,05	33	1	2	1	Trophozoites , entracellular bacteria in cysts
IM 1334	6	4	25,7	7,01	33	1	2	1	Trophozoites , entracellular bacteria in cysts
IM 1558	6	4	22,1	7,17	33	1	2	1	Trophozoites , entracellular bacteria in cysts
IM 1576	6	4	25,9	7,20	33	1	2	1	Trophozoites , entracellular bacteria in cysts
IM 1577	7	4	27,6	7,34	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1335	7	4	25,8	7,09	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1336	7	4	25,8	7,12	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1559	7	4	21,8	7,24	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1560	7	4	22,8	7,25	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1578	7	4	27,6	7,35	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1591	7	4	25,6	7,05	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1592	7	4	25,7	7,12	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1595	8	4	25,4	7,38	33	1	1	1	Trophozoites , extracellular bacteria in cysts
IM 1574	8	4	26,2	7,37	33	1	2	1	Trophozoites , extracellular bacteria in cysts
IM 1331	8	4	24,1	6,95	33	1	2	1	Trophozoites , extracellular bacteria in cysts

Code	Sample Source	Season	Temperature	pH	Incubated	Trophozoites	A Cysts	Round Cysts	Comments
IM 1563	8	4	23,2	7,33	33	1	2	1	Trophozoites , extracellular bacteria in cysts

Seasons: 1: Autumn; 2: Winter; 3: Spring; 4: Summer; Sample Source: 1: Maturation pond, Process 8; 2: Treated sewage, Process 7; 3: Untreated Sewage, Process 1; 4: Sewage entering the bioreactor, Process 2; 5: Bound oxygen available as Nitrate/denitrification zone, Process 4; 6: No oxygen available, Process 3 ; 7: Oxygenated zone/Nitrification zone, Process 5; 8: Bioreactor effluent after nutrient removal, Process 6. All other fields:1: Positive; 2: Negative

Table A-2: ARB's staining and culture results.

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Esherichia coli	*Salmonella*	*Shigella*	*Vibrio Cholerae*	*Legionella* species	M *Avium* complex
IM 1071	1	1	TIBC	2	1	1	37	1	1	1	2	2	1
IM 1073	1	1	TIBC	2	1	1	37	1	2	1	0	2	1
IM 1103	1	1	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1104	1	1	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1072	1	1	IBC	2	2	2	37	1	2	1	0	2	1
IM 1093	1	1	TIBC	2	1	2	37	1	2	1	0	2	1
IM 1094	1	1	TEBC	2	1	2	37	1	2	1	2	2	1
IM 1107	1	1	TEBC	2	1	2	37	1	1	1	2	2	1
IM 1091	1	1	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1105	2	1	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1074	2	1	TIBC	2	1	2	37	1	2	1	0	2	2
IM 1082	2	1	EBC	2	2	2	37	1	2	1	2	2	2
IM 1096	3	1	TIBC	2	1	2	37	1	2	1	0	2	1
IM 1075	3	1	IBC	2	2	2	37	1	2	1	2	2	2
IM 1083	3	1	TIBC	2	1	2	37	1	2	1	2	2	2

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Esherichia coli	*Salmonella*	*Shigella*	*Vibrio Cholerae*	*Legionella* species	M *Avium* complex
IM 1108	3	1	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1076	4	1	IBEB	2	2	1	37	1	2	1	0	2	1
IM 1113	4	1	TEBC	2	1	2	37	1	2	1	2	2	1
IM 1084	4	1	TIBC	2	1	2	37	1	2	1	2	2	2
IM 1090	4	1	TIBC	2	1	2	37	1	2	1	0	2	2
IM 1099	4	1	TIBC	2	1	2	37	1	2	1	0	2	2
IM 1077	5	1	TIBC	2	1	1	37	1	2	1	2	2	1
IM 1111	5	1	TIBC	2	1	1	37	1	2	1	0	2	1
IM 1085	5	1	TIBC	2	1	2	37	1	2	1	0	2	1
IM 1100	5	1	TIBC	2	1	2	37	1	2	1	0	2	2
IM 1078	6	1	TIBC	2	1	2	37	1	2	1	0	2	2
IM 1086	6	1	TIBC	2	1	2	37	1	2	1	2	2	2
IM 1095	6	1	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1112	6	1	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1087	7	1	TIBC	2	1	1	37	1	2	1	0	2	1
IM 1097	7	1	TEBC	2	1	1	37	1	2	1	2	2	1

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Esherichia coli	*Salmonella*	*Shigella*	*Vibrio Cholerae*	*Legionella* species	M *Avium* complex
IM 1079	7	1	EBC	2	2	2	37	1	2	1	0	2	1
IM 1110	7	1	TEBC	2	1	2	37	1	2	1	2	2	1
IM 1088	7	1	IBC	2	2	2	37	1	2	1	0	2	2
IM 1080	7	1	EBC	2	2	2	37	1	2	1	2	2	2
IM 1098	7	1	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1109	7	1	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1106	8	1	TIBC	2	1	1	37	1	2	1	0	2	1
IM 1081	8	1	TIBC	2	1	1	37	1	2	1	2	2	2
IM 1089	8	1	TIBC	2	1	2	37	1	2	1	2	2	2
IM 1092	8	1	TIBC	2	1	2	37	1	2	1	0	2	2
IM 1167	1	2	TEBC	2	1	1	37	1	1	1	2	1	1
IM 1159	1	2	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1160	1	2	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1165	1	2	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1200	1	2	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1187	1	2	TEBC	2	1	2	37	1	2	1	0	2	1

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Escherichia coli	*Salmonella*	*Shigella*	*Vibrio Cholerae*	*Legionella* species	M *Avium* complex
IM 1188	1	2	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1201	1	2	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1158	1	2	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1164	1	2	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1191	1	2	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1202	1	2	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1189	2	2	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1151	2	2	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1197	3	2	TEBC	2	1	2	37	1	2	1	2	2	1
IM 1208	3	2	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1156	3	2	TIBC	2	1	2	37	1	2	1	0	2	2
IM 1166	3	2	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1196	4	2	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1155	4	2	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1163	4	2	TEBC	2	1	2	37	1	1	1	2	2	1
IM 1172	4	2	TEBC	2	1	2	37	1	2	1	0	2	1

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Esherichia coli	Salmonella	Shigella	Vibrio Cholerae	Legionella species	M Avium complex
IM 1206	4	2	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1168	5	2	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1152	5	2	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1193	5	2	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1203	5	2	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1153	6	2	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1207	6	2	TIBC	2	1	1	37	1	2	1	2	2	1
IM 1170	6	2	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1192	6	2	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1169	7	2	TEBC	2	1	1	37	1	1	1	0	1	1
IM 1154	7	2	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1194	7	2	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1204	7	2	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1205	7	2	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1195	7	2	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1157	7	2	TEBC	2	1	2	37	1	2	1	0	2	2

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Escherichia coli	Salmonella	Shigella	Vibrio Cholerae	Legionella species	M Avium complex
IM 1171	7	2	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1162	8	2	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1199	8	2	TEBC	2	1	1	37	1	1	1	2	2	1
IM 1150	8	2	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1190	8	2	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1231	1	3	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1255	1	3	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1256	1	3	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1220	1	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1234	1	3	TEBC	2	1	2	37	1	2	1	2	2	1
IM 1243	1	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1244	1	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1258	1	3	TEBC	2	1	2	37	1	2	1	2	2	1
IM 1221	1	3	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1223	1	3	TEBC	2	1	2	37	1	1	1	2	2	2
IM 1232	1	3	TEBC	2	1	2	37	1	2	1	2	2	2

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Escherichia coli	Salmonella	Shigella	Vibrio Cholerae	Legionella species	M Avium complex
IM 1246	1	3	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1219	2	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1230	2	3	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1242	2	3	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1254	2	3	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1228	3	3	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1239	3	3	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1251	3	3	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1303	3	3	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1240	4	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1304	4	3	TEBC	2	1	2	37	1	2	1	2	2	1
IM 1229	4	3	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1252	4	3	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1235	5	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1247	5	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1224	5	3	TEBC	2	1	2	37	1	2	1	2	2	2

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Escherichia coli	Salmonella	Shigella	Vibrio Cholerae	Legionella species	M Avium complex
IM 1259	5	3	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1248	6	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1225	6	3	TIBC	2	1	2	37	1	2	1	0	2	2
IM 1236	6	3	TEBC	2	1	2	37	1	1	1	0	2	2
IM 1300	6	3	TIBC	2	1	2	37	1	2	1	0	2	2
IM 1226	7	3	TEBC	2	1	2	37	1	2	1	2	2	1
IM 1227	7	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1302	7	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1237	7	3	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1238	7	3	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1249	7	3	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1250	7	3	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1301	7	3	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1245	8	3	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1233	8	3	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1257	8	3	TEBC	2	1	2	37	1	2	1	2	2	1

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Escherichia coli	Salmonella	Shigella	Vibrio Cholerae	Legionella species	M Avium complex
IM 1222	8	3	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1555	1	4	TEBC	2	1	1	37	1	1	1	0	1	1
IM 1556	1	4	TEBC	2	1	1	37	1	1	1	2	1	1
IM 1572	1	4	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1573	1	4	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1588	1	4	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1329	1	4	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1330	1	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1332	1	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1554	1	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1571	1	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1586	1	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1587	1	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1328	2	4	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1570	2	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1579	3	4	TEBC	2	1	1	37	1	2	1	0	2	1

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Escherichia coli	Salmonella	Shigella	Vibrio Cholerae	Legionella species	M Avium complex
IM 1337	3	4	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1593	3	4	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1561	3	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1562	4	4	TEBC	2	1	1	37	1	1	1	2	1	1
IM 1580	4	4	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1585	4	4	TEBC	2	1	2	37	1	2	1	2	2	1
IM 1594	4	4	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1338	4	4	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1553	4	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1575	5	4	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1333	5	4	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1557	5	4	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1589	5	4	TEBC	2	1	2	37	1	2	1	2	2	1
IM 1590	6	4	TIBC	2	1	1	37	1	1	1	2	1	1
IM 1334	6	4	TEBC	2	1	1	37	1	2	1	0	2	1
IM 1558	6	4	TEBC	2	1	1	37	1	2	1	2	2	1

Code	Sample Source	Season	Comment codes	Gram stain	Giemsa stain	Z-N stain	Incubated	Escherichia coli	Salmonella	Shigella	Vibrio Cholerae	Legionella species	M Avium complex
IM 1576	6	4	TIBC	2	1	2	37	1	2	1	0	2	1
IM 1577	7	4	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1335	7	4	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1336	7	4	TEBC	2	1	2	37	1	2	1	0	2	1
IM 1559	7	4	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1560	7	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1578	7	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1591	7	4	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1592	7	4	TEBC	2	1	2	37	1	2	1	2	2	2
IM 1595	8	4	TEBC	2	1	1	37	1	1	2	1	1	1
IM 1574	8	4	TEBC	2	1	1	37	1	2	1	2	2	1
IM 1331	8	4	TEBC	2	1	2	37	1	2	1	0	2	2
IM 1563	8	4	TEBC	2	1	2	37	1	2	1	0	2	2

Seasons: 1: Autumn; 2: Winter; 3: Spring; 4: Summer; Sample Source: 1: Maturation pond; 2: Treated sewage; 3: Untreated Sewage; 4: Sewage entering the bioreactor; 5: Bound oxygen available as Nitrate/denitrification zone; 6: No oxygen available; 7: Oxygenated zone/Nitrification zone; 8: Bioreactor effluent after nutrient removal. All other fields: 0: No Growth; 1: Positive; 2: Negative. Comment codes: TEBC: Trophozoites and cysts with extracellular bacteria; TIBC: Trophozoites and cysts with intracellular bacteria; EBC: Cysts with extracellular bacteria present; IBC: cysts with intracellular bacteria; IBEB: Cysts with intra and extra cellular bacteria.

Table A-3: PCR results

Code (IM)	Amoebae	*Vibrio Cholerae*	*Legionella* species	M *Avium* complex	*Chlamydia*
1071	2	2	2	2	2
1073	2	2	2	2	2
1076	2	2	2	2	2
1077	2	2	2	2	2
1087	2	2	2	2	2
1097	2	2	2	2	2
1103	2	2	2	2	2
1104	2	2	2	2	2
1105	2	2	2	2	2
1106	2	2	2	2	1
1111	2	2	2	2	2
1153	2	2	1	2	1
1154	2	2	2	2	1
1159	2	2	1	2	1
1165	2	2	2	2	2
1167	2	2	1	2	1
1168	2	2	2	2	1
1169	2	2	1	2	1
1189	2	2	2	2	1
1194	2	2	2	2	1
1196	2	2	2	2	1
1199	2	2	2	2	2
1204	2	2	2	2	1
1205	2	2	2	2	1
1206	2	2	2	2	2
1207	2	2	2	2	1
1208	2	2	2	2	2
1228	2	2	2	2	1
1231	2	2	2	2	1
1239	2	2	2	2	2
1245	2	2	2	1	1
1251	2	2	2	2	2
1255	2	2	2	2	2
1256	2	2	2	2	1
1303	2	2	2	2	2
1334	2	2	2	2	1
1555	2	2	1	2	2
1556	2	2	1	2	2
1558	2	2	2	2	1
1562	2	2	1	2	1

Code (IM)	Amoebae	Vibrio Cholerae	Legionella species	M Avium complex	Chlamydia
1572	2	2	2	2	2
1573	2	2	2	2	1
1574	2	2	2	2	1
1575	2	2	2	2	1
1577	1	2	2	2	1
1579	2	2	2	2	2
1580	2	2	2	2	2
1588	2	2	2	2	1
1590	1	2	1	2	2
1595	2	2	1	2	2

1: Positive; **2**: Negative

Appendix B:

B1 PCR Gels

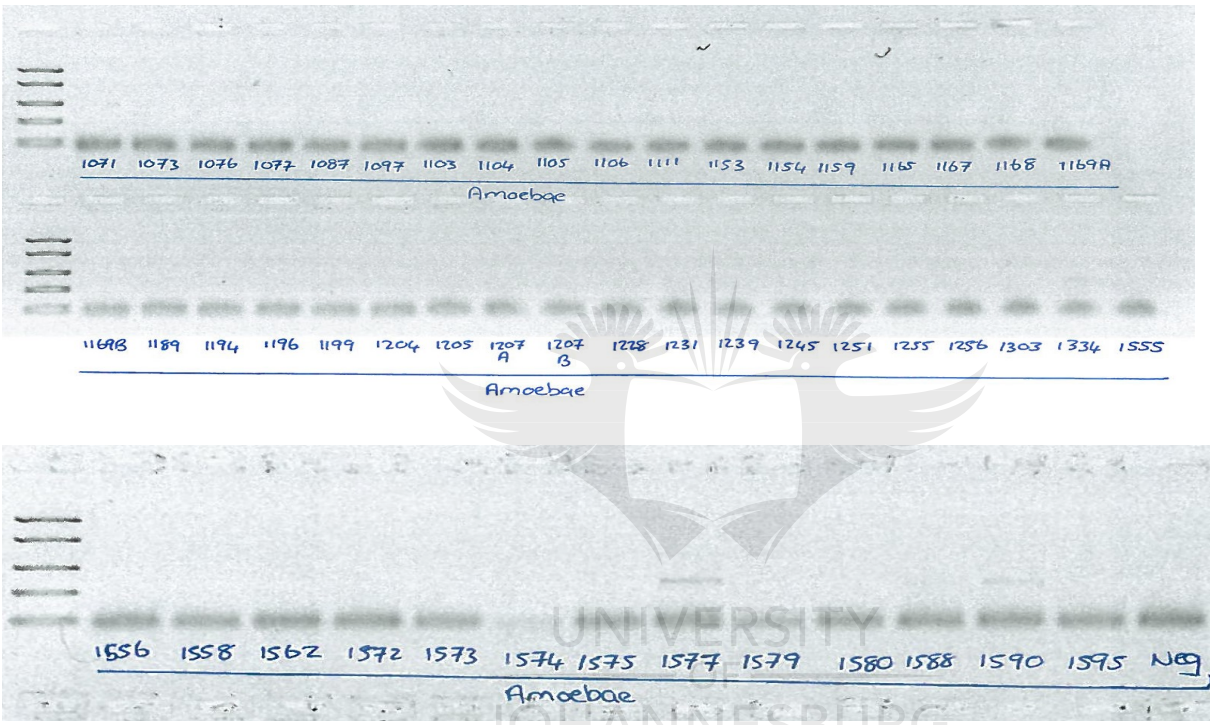


Figure B-1: Amoeba PCR gels

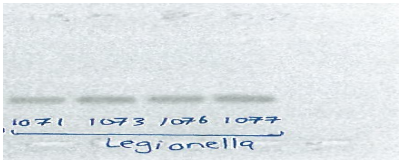


Figure B-2: PCR gels for *Legionella*

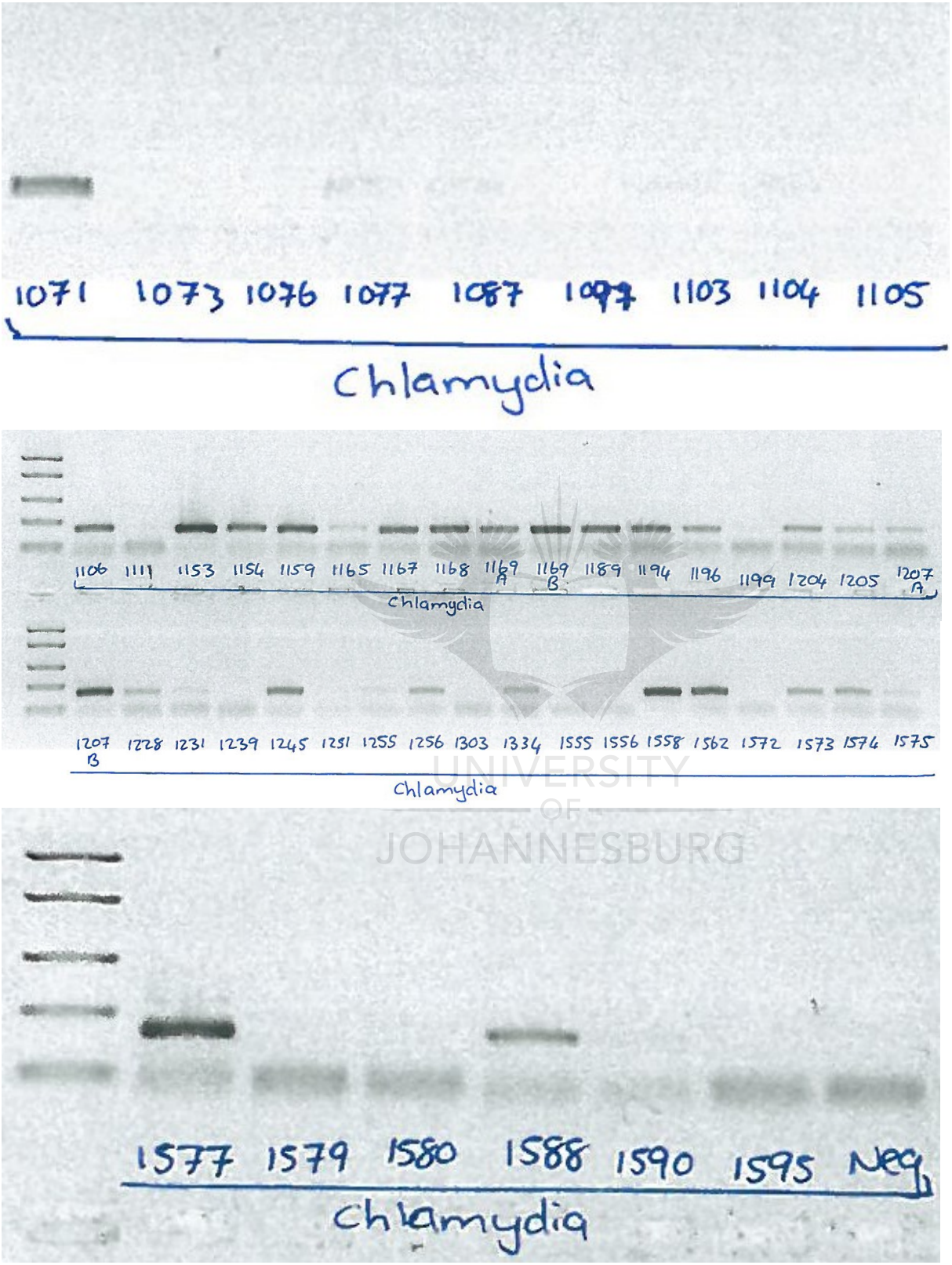
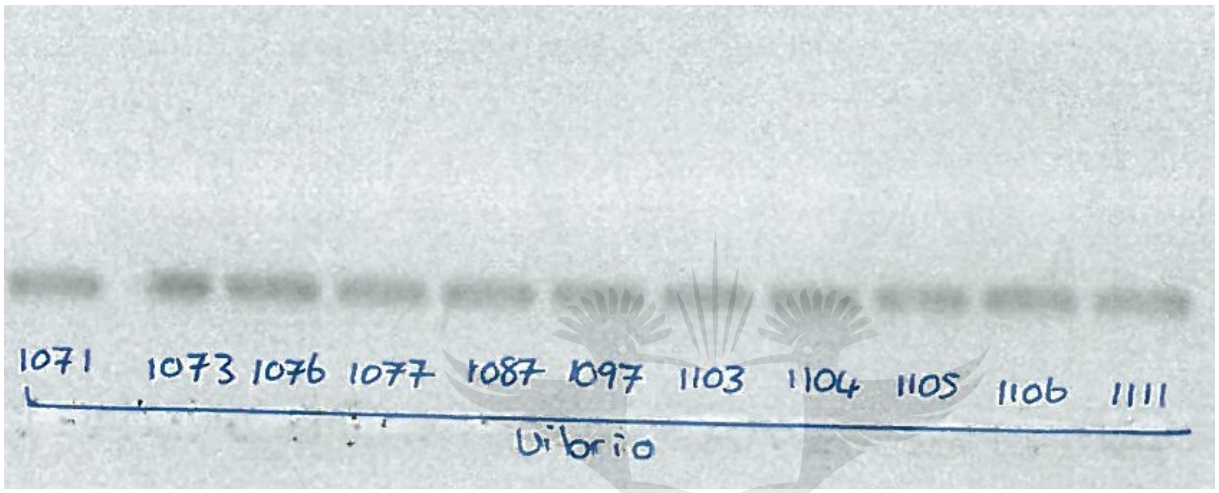
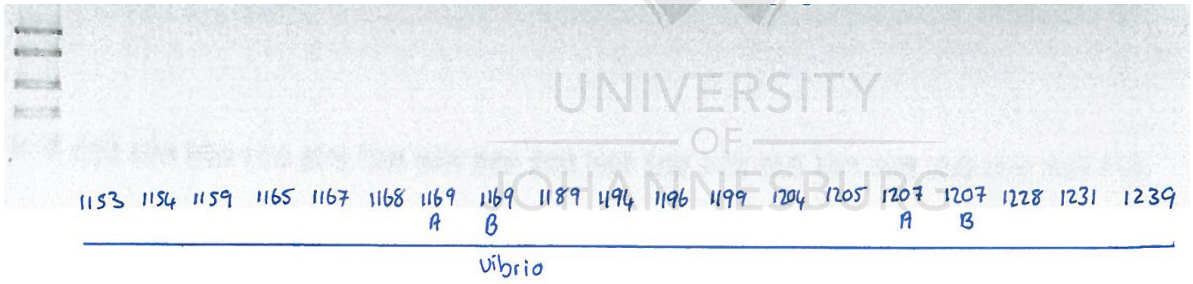


Figure B-3: *Chlamydia* PCR gels





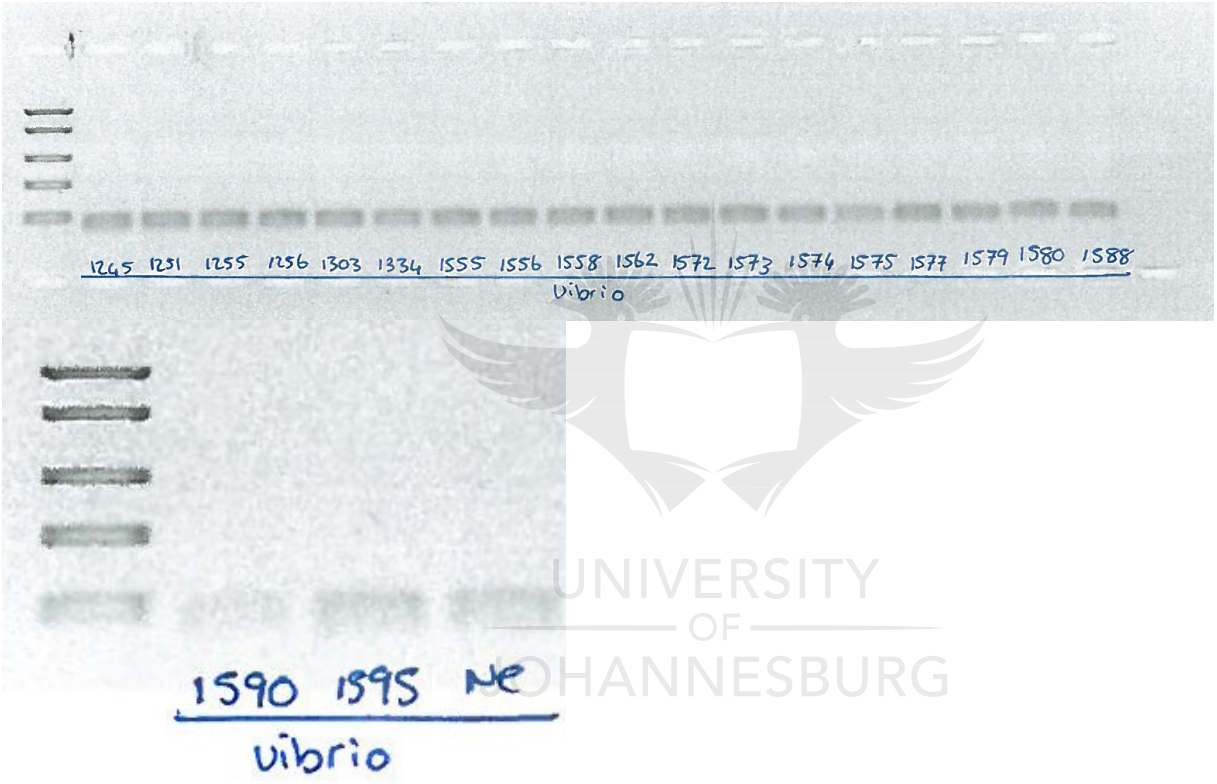


Figure B-4: *Vibrio Cholera* PCR gels

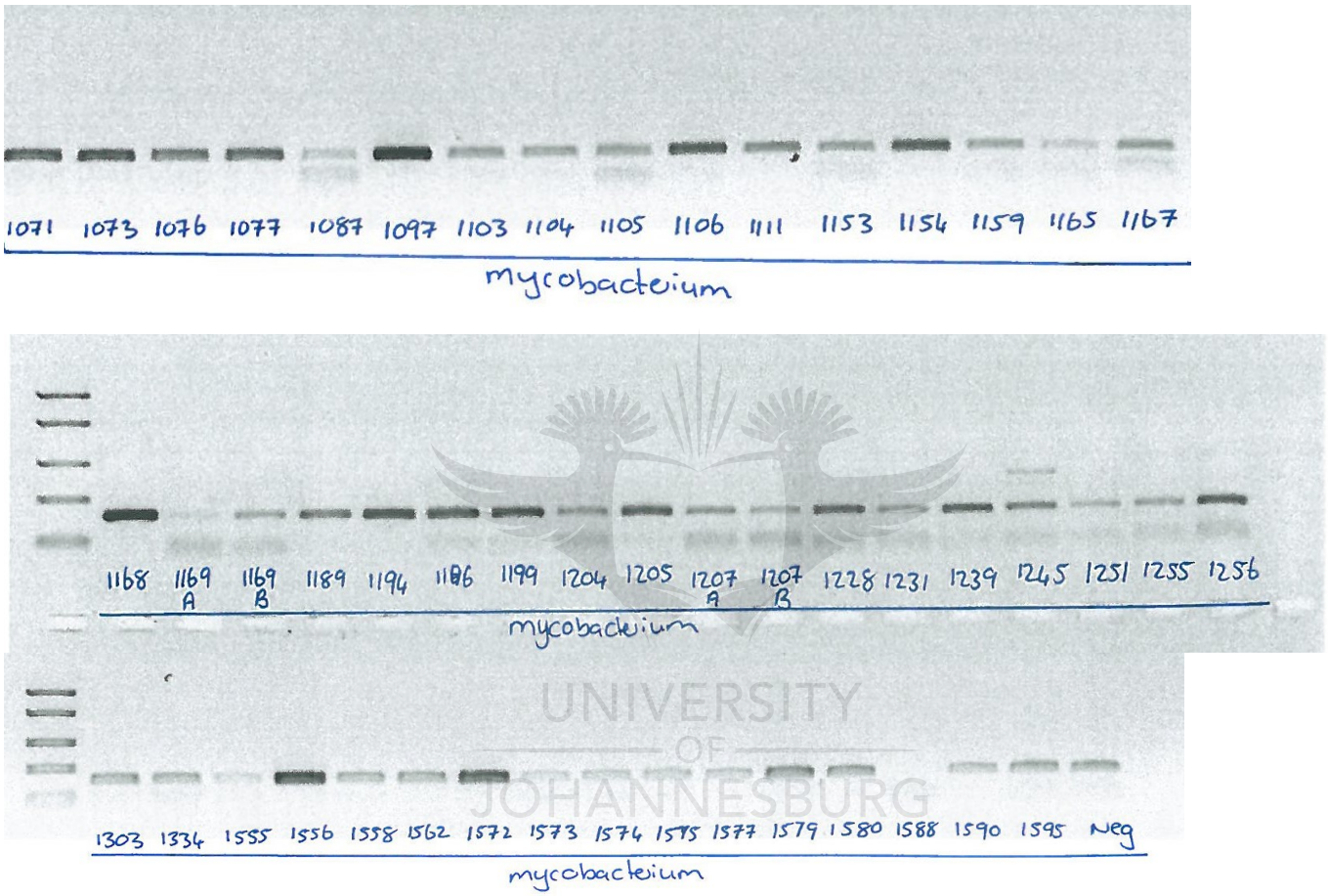


Figure B-5: *Mycobacterium* PCR gels.